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By

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Copepods in aquaculture: Improving the quality and quantity of copepods for application as potential live prey

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Résumé

Cette thèse contient quatre études majeures ciblant l'aquaculture des copépodes, incluant des recherches sur (1) les conditions optimales de la culture de deux espèces tropicales, le calanoide *Acartia bilobata* et le cyclopoide *Apocyclops royi* ; (2) les œufs de résistance d'*A. bilobata* ; (3) les effets d'une sélection d'une souche performante d'*A. royi* en utilisant un nouveau protocole multi-générationnel et thermique ; (4) la pertinence de l'introduction des copépodes dans le régime alimentaire des larves de poissons.

Pour le nourrissage des copépodes, les micro-algues de petites tailles (3-6 µm) riches en acides gras polyinsaturés sont plus avantageuses pour les deux espèces testées dans cette étude. De plus, nous avons démontré que le cyclopoide *A. royi*, contrairement au calanoide *A. bilobata*, est capable de synthétiser des chaines longues d'acides gras polyinsaturés (C20 et C22) à partir de chaines plus courtes d'acides gras polyinsaturés (C16 et C18). La salinité affecte différemment les stades de développement d'*A. royi* mais pour obtenir une croissance maximale de la population il faut cultiver cette espèce à salinité 20. Les différentes caractéristiques de production d'œufs quiescents et de diapause ont été identifiées chez deux souches différentes d'*A. bilobata.* A noter que ces œufs de résistance stockés à faible température (4°C) restent viables pendant plusieurs mois. Concernant la sélection de copépodes sur plusieurs générations, outre le suivi de la fitness des femelles d'*A. royi* le contenu et la composition en acides gras ont été également retenus comme critères de sélection.

Basé sur nos résultats obtenus sur l'élevage de larves de poissons, le régime alimentaire basé sur les nauplii d'*A. tonsa* obtenus en utilisant les œufs de résistance stockés à faible température, pourrait être avantageux pour des larves pélagiques; cependant, l'utilisation d'*A. royi* comme proie vivante devrait être soigneusement évaluée par la considération spécifique du comportement de nage de l'espèce de poisson ciblée. Cette thèse contribue à améliorer nos connaissances biologiques et physiologiques sur les copépodes et présente des implications directes sur leur utilisation comme proie vivante prometteuse pour l'élevage larvaire de poissons.

Mots-clés : copépodes, aquaculture, nutrition, sélection écophysiologique, œufs de résistance, proie vivante, élevage larvaire, acides gras

Abstract

The present thesis contains four major studies addressed on copepod aquaculture, including the investigation of the (1) optimal culture conditions of two tropical copepod species *Acartia bilobata* (Calanoida) and *Apocyclops royi* (Cyclopoida); (2) resting eggs of *A. bilobata*; (3) effects of selective breeding on *A. royi* by using a new multigenerational and thermal protocol; (4) feeding suitability of copepod-based diets on larval fish.

For copepod feeding, small-sized microalgae (3-6 μm) with rich polyunsaturated fatty acids (PUFAs) are preferable for both copepod species. Furthermore, we demonstrated that the cyclopoid *A. royi*, contrary to the calanoid *A. bilobata*, is capable of synthesizing long chains of polyunsaturated fatty acids (PUFAs) (C20 and C22) from shorter chains of PUFAs (C16 and C18). The salinity affects differently the life cycle development of *A. royi*, but to obtain the maximum population growth it is necessary to cultivate this species at salinity 20. Various characteristics of quiescent and diapausing eggs productions are identified in two culture strains of *A. bilobata* with different domestication levels, and those eggs remain viable after several months of cold storage (4°C). The larger female with higher nauplii production and compensative fatty acid increase were obtained in the cold-selected *A. royi* culture strain.

Based on our results found in fish larvae feeding experiments, the diet based on *A. tonsa* nauplii derived from quiescent eggs could be beneficial for pelagic fish larvae; however, the use of *A. royi* as live prey should be carefully evaluated by the specific consideration of swimming behavior of targeted fish species. This thesis reveals the biological and physiological understandings on copepods, and provides implications on the application of copepods as promising live prey for larviculture.

Key words：Copepod, Aquaculture, Nutrition, Selective breeding, Resting egg, Live prey, Larviculture, Fatty acids

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Chapter 1. General introduction

1.1. Thesis outline

The cooperation program between National Taiwan Ocean University (NTOU) and University Lille 1 - Science and Technology (USTL) is coordinated by Professor Jiang-Shiou Hwang and Professor Sami Souissi. Four co-tutorial Ph.D. students have already obtained double Ph.D. degree from NTOU and USTL. The bilateral collaboration contributed significantly to strengthen the collaboration between Taiwan and France in the field of marine biology and several applications (i.e. ecotoxicology and aquaculture). This thesis aims to apply the knowledge on life cycle, physiology and reproductive biology of copepods to improve marine larviculture. A part of this study was performed in a partner Aquarium (Centre National de la Mer, Nausicaa Aquarium) of USTL, and it was contributed to the COPEFISH project. This pilot project was endorsed by the Competitively Pole AQUIMER in Boulogne sur Mer and co-funded by the 'Conseil Régional Nord Pas de Calais' as an emergent research program.

1.2. Copepods in aquaculture

The capture marine fishery resources have been heavily exploited to meet the ever-increasing human demand for the emerging protein resources from fish. The dramatically boosting fish consumption has resulted in global urbanization, rapid human population growth, facilitated systematic shipment of fishery product and advanced fishing techniques. The overfishing effect has negatively impacted the marine biodiversity. Besides the food consumption, ornamental fish have also faced the stress of overcapture. The markets of aquarium trade extremely depend on the wild-collected fish from coral reefs or tropical lagoons. To maintain sustainability of fishery resources, captive breeding of fish is believed to be a promising solution which

can help reduce the capture stress. The research for aquaculture are necessary to be invested and focused in different directions.

Between 1980 and 2012, world overall production volume in aquaculture has increased at an average rate of 8.6 percent per year (SOFIA Report FAO, 2014; Fig. 1.1). Though aquaculture is a fast-growing industry, the development is still limited by the technical obstacle to properly control the early life stages of many emerging farmed fish species. Particularly, the significant difficulty is considered the failure of first feeding of very young larvae. Larvae deplete their yolk reserves few days after they hatch from eggs, larval fish do not benefit from a well-developed digestive system to efficiently digest the formulated diets.

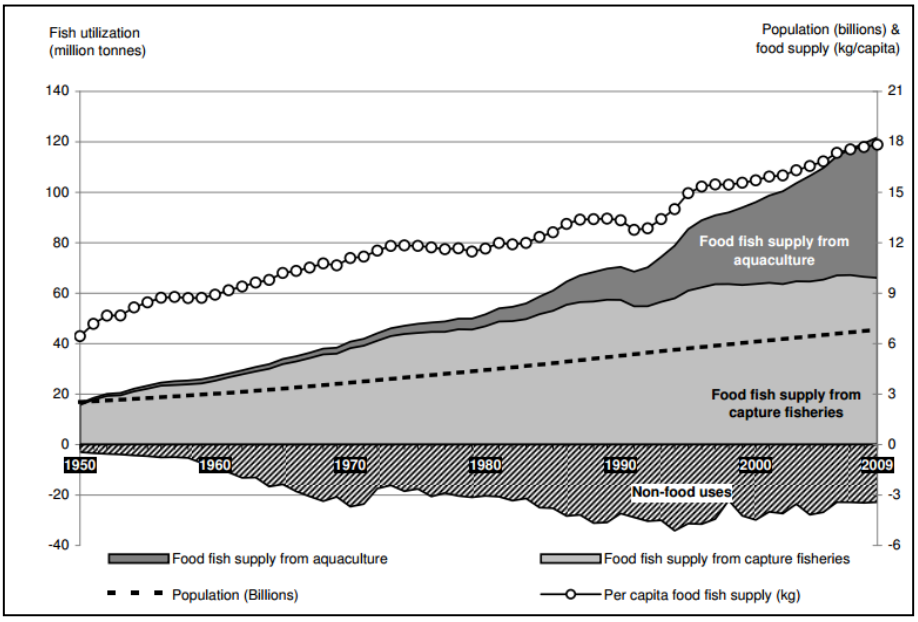

Fig. 1.1 World fish utilization and supply. Credit: SOFIA Report FAO 2014

Contrary to formulated diets, live feeds swim into the water column and are constantly available for larvae (Lee et al., 2010; Wu et al., 2011). This feature triggers the higher feeding response in the larval stage of most fish species. The commonly selected live

prey for the larval production in aquaculture industry, including microalgae, rotifers, *Artemia* and copepods (Conceição et al., 2010; Cheng et al., 2011; Kumar et al., 2012; Mahjoub et al., 2012). Rotifers and *Artemia* are easy to be produced in high densities and they are also highly available, however, due to their incomplete nutritional profile, they are usually required to be enriched with commercial products to enhance their nutritional quality (Sargent et al., 1997).

Copepods are the largest and most diversified fauna in crustaceans, and they are the most numerous metazoans in aquatic communities. The 11500 currently-known species of copepods is potentially underestimated (Humes, 1994). In marine ecosystems, copepods play important roles of energy linkages across trophic levels, copepods are the natural prey for the larvae of many marine fish species (Støttrup, 2000). In particular, their larval stages – nauplii, are considered the much appropriate and balanced diet for marine fish larvae. Several research programs have demonstrated the benefits of using copepods as live food in larviculture. We summarize here the advantages of copepod supplementary diets in culturing fish larvae:

- Prey size is a paramount factor for planktivorous larval fish (Brooks & Dodson, 1965), many copepods offer a great size suitability (<100μm) during one or more developmental stages for small larval fish which have small mouth gape size (Payne & Rippingale, 2000; McKinnon et al., 2003).

- The swimming patterns of some copepod species can trigger higher feeding response in larval fish (Støttrup, 2000; Buskey, 2005).

- Naupliar copepods are much easier to be digested by fish larvae in comparison to rotifers and *Artemia* (Pedersen, 1984; Schipp, 2006).

- Copepods have a higher nutritional value than rotifers and *Artemia* (Watanabe et al., 1983; Drillet et al., 2006b). In fact, they are an excellent source of Highly Unsaturated Fatty Acids (HUFAs) in their polar lipid fraction. This characteristic makes copepod lipid more biologically available to fish larvae (Evjemo & Olsen, 1997). Additionally, copepods are also a good source of vitamin A (Rønnestad et al., 1998), vitamin C, vitamin E (Schipp, 2006) and minerals (Watanabe et al., 1983).

A number of fish species require copepod supplementary diets to achieve their improved development and survival in experimental captive breeding. The beneficial effects of copepod-supplementary diets have been revealed in many larval feeding studies, for instance, better survival, pigmentation and retinal development of halibut larvae *Hippoglossus hippoglossus* (Shields et al., 1999), increased growth and survival rate of West Australian dhufish *Glaucosoma hebraicum*, pink snapper *Pagrus auratus*, fat snook *Centropomus parallelus*, gilt-head sea bream *Sparus aurata* and dusky grouper *Epinephelus marginatus* (Payne et al., 2001; Russo et al., 2009; Barroso et al., 2013; Piccinetti et al., 2014). Moreover, the copepod supplementary diets have also promoted the successful captive breeding of marine ornamental fish, such as seahorses *Hippocampus subelongatus*, *H. reidi* and *H. kuda* (Payne & Rippingale, 2000; Souza-Santos et al., 2013; Thuong & Hoang, 2015), clown fish *Amphiprion clarkii* (Olivotto et al., 2010), lemonpeel angelfish *Centropyge flavissimus* (Olivotto et al., 2006), cardinal fish *Apogon quadrifasciatus* (Saravanan et al., 2013) and damselfish

Dascyllus trimaculatus, *D. aruanus* and *Pomacentrus caeruleus* (Gopakumar & Santhosh, 2009).

Although copepods are favourite candidates as live food in marine larviculture, the difficulties in their optimal culture as well as the poorly addressed information about the biological characteristics among various species do not encourage the industry of aquaculture to extensively use copepods as live feeds. Recently, many studies have been carried out to investigate the optimal conditions of culturing emerging local copepod species in different regions in the world. Indeed, as diverse taxon, the appropriate culture condition for copepod is species specific. Several parameters could affect the productivity of copepod culture, e.g., salinity, temperature, diet density and the genetic quality of various culture strains. To discover the culture methods and identification of the optimal conditions for improving the productivity of novel copepod species at a regional scale can help enhance the fishery production from aquaculture. Therefore, this thesis aimed to investigate selective biological traits of different species of copepods under different culture conditions.

1.3 Selected copepod species in this study

Three species of copepods were selected from Taiwan and France. On Taiwanese side, pure strain of *Acartia bilobata* (Fig. 1.2) and *Apocyclops royi* (Fig. 1.3) were originally isolated from brackish aquaculture ponds in southern Taiwan. On French side, the long-term acclimated culture strain (>20 years) of *Acartia tonsa* (Fig 1.4) is originally isolated from coastal water in Denmark.

A. bilobata (female size: 0.95-1.10 mm, male size: 0.90-0.95 mm, egg size: 85-90 μm) is a planktonic free-spawning tropical calanoid species, and it offers the feasibility of egg collection for the investigations addressed on copepod eggs. *A. royi* (female size: 0.75-0.85 mm, male size: 0.65-0.75 mm, egg size: 80-85 μm) is a semi benthic tropical cyclopoid species, and it is commonly harvested from out-door ponds for feeding larvae or postlarvae of groupers (*Epinephelus* spp.) at Taiwanese hatcheries.

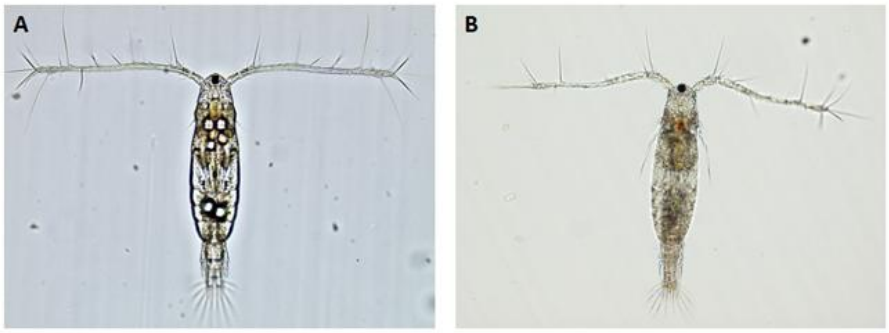

Fig. 1.2 Adult *Acartia bilobata* (A) female (B) male

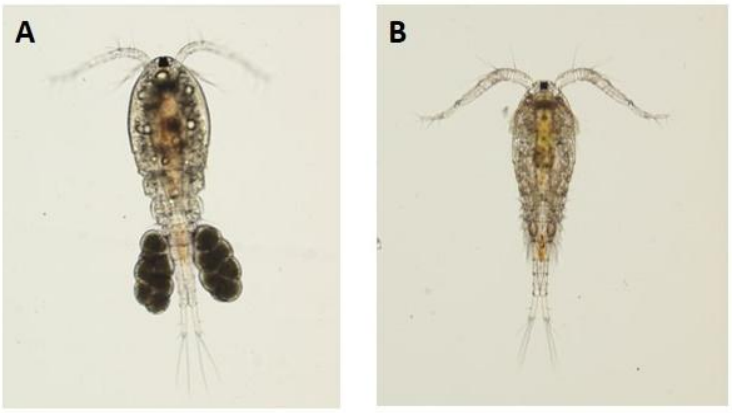

Fig. 1.3 Adult *Apocyclops royi* (A) female (B) male

A. tonsa (female size: 1.20-1.30 mm, male size: 1.10-1.20 mm, egg size: 90-100 μm) is a common planktonic free-spawning calanoid species in temperate regions. Their eggs can hatch after a storage period in cold condition. Therefore, the cold-stored eggs arise a great interest for larval feeding.

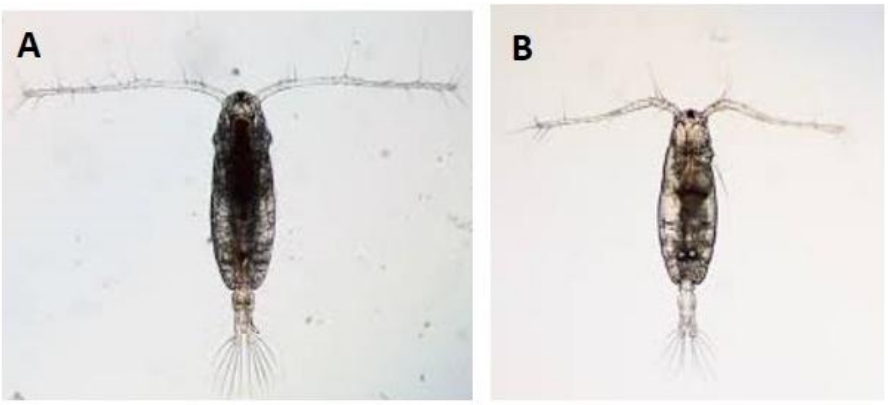

Fig. 1.4 Adult *Acartia tonsa* (A) female (B) male

1.4 Research framework and Objectives

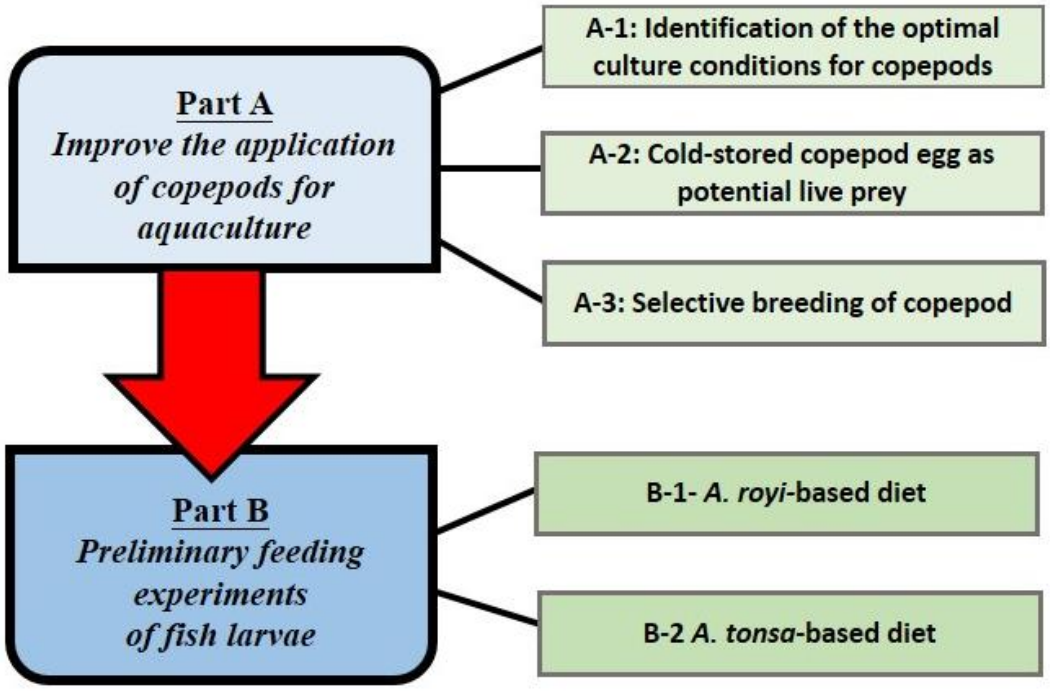

Fig. 1.5 The research framework of present Ph.D. thesis

Objective:

- **A-1** Identification of optimal culture conditions of copepods: investigate the productive patterns of *A. bilobata* and *A. royi* in different culture conditions (salinity, photoperiod and diet)

- **A-2** Cold-stored copepod egg as potential live prey: investigate the viability of cold-stored egg of *A. bilobata* to promote a novel live prey candidate for tropical hatchery.

- **A-3** Selective breeding of copepod: improve the aquaculture potential of *A. royi* through thermal selective breeding.

- **B-1** *A. royi*-based diet: investigate the feeding suitability of *A. royi* on batfish *Platax orbicularis* and seahorse *Hippocampus reidi* larvae.

- **B-2** *A. tonsa*-based diet: investigate the feeding suitability of *A. tonsa* nauplii derived from cold stored eggs on gilt-head bream *Sparus aurata* larvae.

Chapter 2. The effects of algal diets and salinities on the production of tropical cyclopoid copepod *Apocyclops royi*

* Pan, Y. J. *, Souissi, A., Souissi, S., & Hwang, J. S. (2016). Effects of salinity on the reproductive performance of *Apocyclops royi* (Copepoda, Cyclopoida). Journal of Experimental Marine Biology and Ecology, 475, 108-113.

Pan, Y. J., Sadovskaya I., Hwang, J. S., & Souissi, S. (in prep.) Assessment of the fecundity, population growth and fatty acid composition of *Apocyclops royi* (Cyclopoida, Copepoda) fed on different microalgal diets.

2.1 Literature review

Copepods are the major components in zooplankton community, and most of the copepod species play roles of key trophic linkages between primary producers and higher consumers in the marine ecosystem (Støttrup, 2000; Turner, 2004). In field, the biomass and species composition of copepods significantly alter with the fluctuation of environmental parameters regarding the seasonality and climatic changes (Thomas & Nielsen, 1994; Speirs et al., 2006; Sun et al., 2013). In particular, salinity is one of the most important parameters influencing the ecological and biological responses of copepods. Salinity changes may put additional physiological stresses on copepod and ultimately result in mortality, unsustainable population growth and functional shifts in food webs (Kaartvedt & Aksnes, 1992; Soetaert & Herman, 1994). Although euryhaline copepods have the great capacity to regulate the osmotic stress and survive in a wide range of salinity conditions, as the osmoregulators, euryhaline copepods have to reallocate their metabolic energy due to the increased energy requirement of performing ionic regulation which at least maintains the survivorship of individual (Schmidt-Nielsen, 1997).

Considering the energy allocation in different physiological needs, it can be expected that copepods may change their biological processes among various salinities. A number of studies have reported that the fluctuations of salinity can markedly affect many euryhaline copepods. In the studies of tropical estuarine copepod *Pseudodiaptomus annandalei*, salinity treatments showed significant influences on their reproduction, lifespan and survival rate (Chen et al., 2006; Beyrend-Dur et al., 2009). *Eurytemora affinis*, as a dominant estuarine copepod in Europe and North America, is reported to present different physiological responses among salinities, such

as naupliar survival and development (Devreker et al., 2004), fecundity (Devreker et al., 2009), enzymatic expression (Cailleaud et al., 2007), life cycle (Beyrend-Dur et al., 2009) and swimming behavior (Michalec et al., 2010). The remarkable salinity effect have been also revealed in the studies on different species of genus *Acartia* worldwide. The Baltic *A. tonsa* showed variable egg hatching rate and egg production in different salinities (Holste & Peck, 2006; Peck & Holste, 2006). Chinnery and Williams (2004) reported that the salinity treatments affected egg hatching rate and naupliar survival of four temperate *Acartia* species collected from Southampton Water, UK. In the report from Australia, the different results of population growth and egg hatching rate were found on the tropical copepod *Acartia sinjiensis* cultured in different salinities (Milione & Zeng, 2008).

On the other hand, selecting a suitable food is another important factor that extensively influences the productivity and quality of copepod culture (Kleppel et al., 2005). Recent studies have been focused on the evaluation of suitable microalgal diet on copepods belong to different orders in captive conditions, such as Calanoida (Milione et al., 2007; Camus et al., 2009; Ohs et al., 2010; Pan et al., 2014), Harpacticoida (Pinto et al., 2001; Caramujo et al., 2008; Rajthilak et al., 2014) and Cyclopoida (Lee et al., 2006; Rasdi et al., 2015). The differentiation in the preferences of microalgal food among copepod species may result in many factors, for instance, size and shape suitability, digestibility and nutritional fitness of the microalgae for copepods.

The majority of research mentioned above have been mostly addressed on calanoid and harpacticoid copepods, less effort has been placed on the cyclopoid species (Lee et al.,

2006). The copepod species *A. royi* belongs to Cyclopoida and it can be found in estuaries, coastal waters and brackish aquaculture ponds in subtropical and tropical regions. Moreover, *A. royi* is a food supplement for feeding larval groupers at commercial hatcheries in Taiwan (Su et al., 1997). Like other cultured copepods, they are mostly harvested from outdoor ponds (Liao et al., 2001) where large numbers of copepods can be rapidly harvested at relatively low cost. However, the salinity and food resources may shift dramatically in the outdoor aquaculture ponds due to unstable climatic events, and it causes obstacles for supplying stable copepod production for feeding larval fish in the desired time. Therefore, to optimize the aquaculture potential of *A. royi* in the environment-controlled culture condition, we carried out two studies to investigate the salinity and dietary effects on the reproductive performances of *A. royi*.

Additionally, in order to develop a simplified and accurate method for fatty acid analysis, we conducted an experiment to assess the simplified protocol for the preparation of copepod fatty acid methyl esters (FAMEs). On the basis of the small surface volume of extract substrates, the total lipid extraction of Folch (1957) method could be omitted in the FAMEs preparation of microalgae samples (Gnouma et al., unpublished data). Here, we therefore examine the efficiency of simplified FAMEs preparation method for copepod samples.

2.2 Material and Method

2.2.1 Copepod and microalgae stock culture

Pure strains of copepod *A. royi* and marine microalgae *Isochrysis galbana* (ISO; Haptophyceae), *Tetraselmis cui* (TET; Prasinophyceae) and *Nannochloropsis oculata* (NAN; Eustigmatophyceae) were obtained from Tungkang Biotechnology Research Center of Taiwan. Copepods were cultivated in 20 L polycarbonate carboy with the aerated salinity-adjusted seawater at salinity 20 (mixture of distilled water and Whatman GF/F filtered natural seawater). The cultures were placed in the indoor culture room where was maintained in a photoperiod of 12h: 12h/ light: dark cycle and temperature at 25-28 °C. The culture water was completely changed every week. Batch cultures of algae were performed in 2-L flasks contained Whatman GF/C-filtrated and autoclaved natural seawater supplied with Walne's medium (Walne, 1970). The algae used for copepod feeding was in the exponential growth phase (around 3-4 days after inoculation). ISO was introduced every 2 days at the approximately cell concentration of 10^5 cells/ ml in the stock culture water of copepod, this feeding level was determined to be the sufficient concentration for *A. royi* due to the existence of live algal cells before every feeding.

2.2.2 Assessment of simplified methodology of copepod fatty acid analysis

FAMEs preparation

Mixed-stage *A. royi* were collected from a stock culture tank by sieving the culture water on a 38-µm mesh, specimens were concentrated in the cryovials and conserved at

-80 °C. The samples were then lyophilized and ready for the further protocols. Two protocols were tested to compare their difference (Fig. 2.1). In protocol A (common method), the total lipid was extracted by the Folch method (Folch, 1957) prior to the transesterification reaction. In protocol B (simplified method), the samples were directly conducted with transesterification reaction.

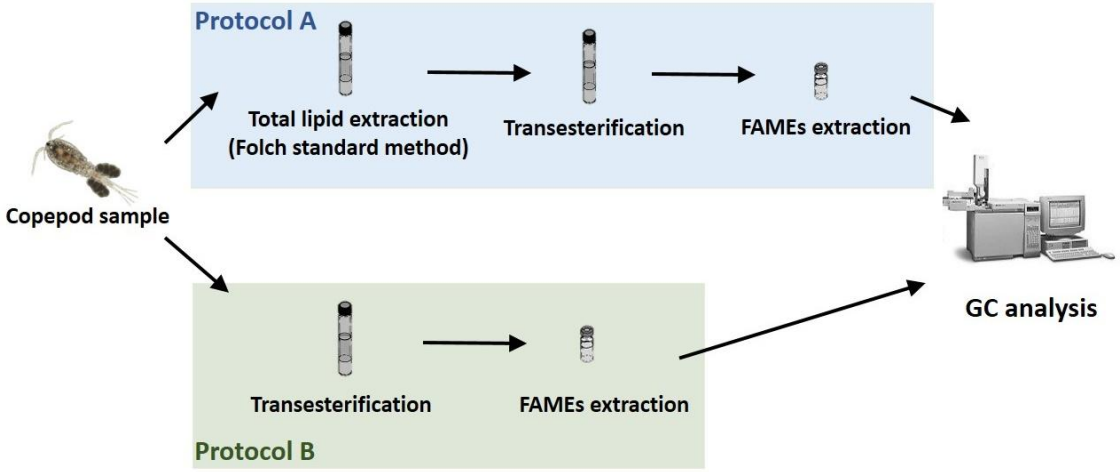

Fig. 2.1 Illustration of FAMEs preparation protocol.

Protocol A: common method

(1.) Around 1 mg of lyophilized *A. royi* was placed each in the 10 ml glass tube (n=3) with 2 ml chloroform/ methanol solvent (volume ratio, 2:1). The tubes were left at -20 °C for a 24-h lipid extraction.

(2.) The samples were then sonicated in ice bath for 2 hours. After sonication, 100 µl of C17 internal (concentration: 0.2 mg/ml) was added in each tube.

(3.) Place the tubes on the aluminum heating block preheated to 60°C. Evaporate the chloroform/methanol solvent by applying nitrogen stream until completely dry.

(4.) Add 2 ml transesterification reagent (MeOH: Toluene: AcOCl, volume ratio: 85:66:15) in the tube, then heat it at 95 °C for 2 h. Cool down the tubes until 60 °C, and apply the nitrogen stream to dry out completely the solvent.

(5.) Add 1 ml hexane in the tube to solubilize the FAMEs, then wash with distilled water. Collect the organic (upper) layer and transfer it in a new tube.

(6.) Repeat the previous step to re-extract again the potential remaining FAMEs in the original tubes, collect the organic layer and place it with the previous collection.

(7.) Wash the total upper layer again with 2 ml distilled water to obtain further purified FAMEs, then filter the organic layer through cotton-plugged Pasteur pipette into a GC vial.

(8.) Completely evaporate the solvent by a nitrogen stream, and solubilize the FAMEs in 100 µl of hexane. Samples are conserved at -20 °C until the GC-MS injection.

Protocol B: Simplified method

(1.) Around 1 mg of lyophilized *A. royi* was placed each in the 10 ml glass tube (n=3) added with 100 µl of C17 internal (concentration: 6mg/ 30ml). Evaporate the solvent of internal standard by applying gentle nitrogen stream till completely dry.

(2.) The sample was proceeded as the step 4 to 8 described in *Protocol A*.

GC-MS condition and data analysis

One microliter of prepared copepod FAMEs was injected into a Trace GC ULTRA system (Thermo Scientific) equipped with a capillary column NMTR-5MS (30 m × 0.25 mm) using a temperature gradient of 170 °C (3 mins) → 250 ◦C at 5 °C / min and with a DSQ II MS detector. The content of fatty acid was calculated as:

$$\text{Fatty acid content } (\mu g/mg) = \frac{\text{Amount of internal standard } (\mu g) \text{x Area of sample fatty acid peak}}{\text{Area of internal standard x Weight of tissue } (mg)}$$

2.2.3 Salinity experiment

Pre-cultivation and salinity acclimation

The pre-cultivation was carried out to standardize the age of copepods used in our experiments. Nauplii were isolated from the stock culture tank and transferred in a new culture tank maintained in the same condition. We observed the culture every day, the copepods were collected one day after the appearance of the first ovigerous female. In order to avoid the mortality caused by acute salinity shock, we conducted the salinity acclimation 1 hour prior to the experiment, approximately 300 adults were collected from the pre-cultivation tank and placed in the 500 ml beaker with 50 ml original culture water (salinity 20). The filtered natural seawater (salinity 35) or distilled water was gradually added into the beakers until the salinity approached (±1 salinity) to the designed salinities (salinity 0, 5, 10, 15, 20, 25, 30, 35). After the salinity acclimation, alive individuals were randomly selected for population and individual experiments.

Population experiment- population growth and clutch size

Eight ovigerous females were randomly collected from the acclimation beakers and transferred in the 1-L beakers with 800 ml water at the designed salinities (salinity 0, 5, 10, 15, 20, 25, 30, 35). The cultures were kept in the thermostatic incubator (MLR-351H; SANYO, Osaka, Japan) programmed at 28 °C and in a photoperiod of 12 h: 12 h light: dark cycle. To maintain the stability of salinity, the algal diets (ISO) were centrifuged (4000 rpm, 1 min) to remove the original culture medium. The water at

different salinities was added for suspending the algal cells, and the prepared algal diet was then introduced to the experimental cultures at the approximate density of 10^5 cells/ ml. Four replicates were examined for each salinity. After 14-day cultivation, the copepod cultures were terminated by 4 % buffered formalin solution. The number of different developmental stages (nauplii, copepodite, male, female and ovigerous female) was counted under the stereomicroscope (OLYMPUS IX71, Tokyo, Japan). After the counting, 20 to 30 ovigerous females, which carried complete egg sacs were randomly sorted from replicates. The ovigerous females were placed on a petri dish, the egg sacs were carefully dissected to examine the clutch size (egg per clutch).

Individual experiment - nauplii production and clutch production

Twelve pairs of adult *A. royi* were sorted from the acclimation beaker and independently cultivated in the 6-well culture plates at designed salinities (salinity 0, 10, 20, 30). The cultures were maintained as the same condition as in the population experiment. Nauplii production and clutch production were recorded by observing every day the culture wells under the stereomicroscope (OLYMPUS IX71, Tokyo, Japan) for 14 days.

2.2.4 Algal diet experiment

Algal diet

Seven treatments of algal diets were designed: 3 single-species diets (ISO, NAN and TET), 3 two-species diets (ISO+NAN, ISO+TET, and TET+NAN) and 1 three-species diet (ISO+NAN+TET). The algal densities were modified from the sufficient feeding level for tropical copepod used by Milione & Zeng (2007), and the cell densities of

each dietary treatment were equivalent to the similar ash-free dry weight. The two-species diets were designed as half of the concentration of the single-species diets of each species, and the three-species diet was composed of a one-third concentration of each species. The algae concentrations were determined by a hemocytometer before the diets were fed to the copepods.

The concentrations of each treatment were as follows: ISO: 100 000 cells ml^{-1}, NAN: 100 000 cells ml^{-1}, TET: 7000 cells ml^{-1}, ISO + NAN: 50 000 + 50 000 cells ml^{-1}, ISO + TET: 50 000 + 3500 cells ml^{-1}, NAN + TET: 50 000 + 3500 cells ml^{-1}, and ISO + NAN + TET: 35 000 + 35 000 + 2 400 cells ml^{-1}. To prepare algal diets, the required volume of algal liquid was centrifuged (4000 rpm, 2 min) to remove the original culture water. The diluted seawater (salinity 20) was added to make the suspensions, and the prepared algal diets were subsequently introduced in the copepod cultures.

Population growth

In order to avoid the resident algal dietary effects on experimental individuals. The culture water was completely exchanged and starved 1 day before the experiment started. After the starving period, eight ovigerous females were randomly collected from the stock culture tank and transferred in a 500 ml beaker with 400 ml diluted seawater (mixture of Whatman GF/C filtered natural seawater and distilled water) at salinity 20. Eight replicates were carried out for each treatment, all the cultures were kept in the thermostatic incubator (MTI-03, Firstek Scientific, Taipei, Taiwan) programmed at 28 °C and in a photoperiod of 12 h: 12 h light: dark cycle. After 14-day, 4 beakers of copepod cultures were fixed by 4% buffered formalin solution. The number of different developmental stages (nauplii, copepodite, male, female and

ovigerous female) was counted under the stereomicroscope (OLYMPUS SZX16, Tokyo, Japan). For examining the fecundity of females fed on different diets, 15 to 20 ovigerous females, which carried complete egg sacs were randomly sorted from every replicate. Those ovigerous females were placed on a petri dish, their egg sacs were carefully dissected to estimate the clutch size (eggs per clutch). The other 4 beakers of copepods were concentrated in the cryovials and conserved at -80 °C for fatty acid analysis.

Fatty acids analysis

The fatty acid analysis of microalgae and copepod samples were performed by the simplified method described in section 2.2.2. Briefly, quantified lyophilized copepod and microalgae were conducted with transesterification, and the FAMEs were solubilize in Hexane. Trace GC ULTRA system (Thermo Scientific) was used to identify the fatty acids.

2.2.5 Data analysis

All statistical analyses of this study were run using SPSS program (SPSS, Chicago, IL, USA). The independent-samples t-test was used to compare the means of actual content of key fatty acid groups between two methods of FAME preparation, and the significance level was designed at $p < 0.05$. Dietary and salinity effects on productive traits were analyzed using one-way ANOVA to compare the mean values. Since the significant differences were detected in all treatments ($p < 0.05$); Tukey's multiple comparison test was then used to analyze specific differences between pairs of treatments.

2.3 Result

2.3.1 Assessment of simplified methodology of copepod fatty acid analysis

The actual content of key fatty acid groups of *A. royi* extracted by different protocols were summarized in Fig. 2.2, and Table 2.1 shows the fatty acid composition. In the common method, the contents of various fatty acids are as follows: saturated fatty acids (SFA): 46.5 ± 7.3 μg/mg DW, monounsaturated fatty acids (MUFA): 16.3 ± 3.7 μg/mg DW, polyunsaturated fatty acids (PUFA): 71.2 ± 25.2 μg/mg DW, eicosapentaeonic acid (EPA): 5.3 ± 0.8 μg/mg DW, docosahexaneoix acid (DHA): 16.7 ± 4.3 μg/mg DW, omega-3 polyunsaturated fatty acids (n-3 PUFA): 55.5 ± 22.4 μg/mg DW, omega-6 polyunsaturated fatty acids (n-6 PUFA): 15.6 ± 2.9 μg/mg DW. In the simplified method, the contents of various fatty acids are as follows: SFA: 36.2 ± 5.6 μg/mg DW, MUFA: 18.3 ± 3.3 μg/mg DW, PUFA: 60.1 ± 9.9 μg/mg DW, EPA: 4.9 ± 1.0 μg/mg DW, DHA: 13.6 ± 2.08 μg/mg DW, n-3: 44.4 ± 7.6 μg/mg DW, n-6: 15.6 ± 2.3 μg/mg DW. This preliminary experiment confirmed that the prior lipid extraction with chloroform-methanol do not significantly improve the yields of fatty acids, we therefore performed the simplified protocol in this thesis.

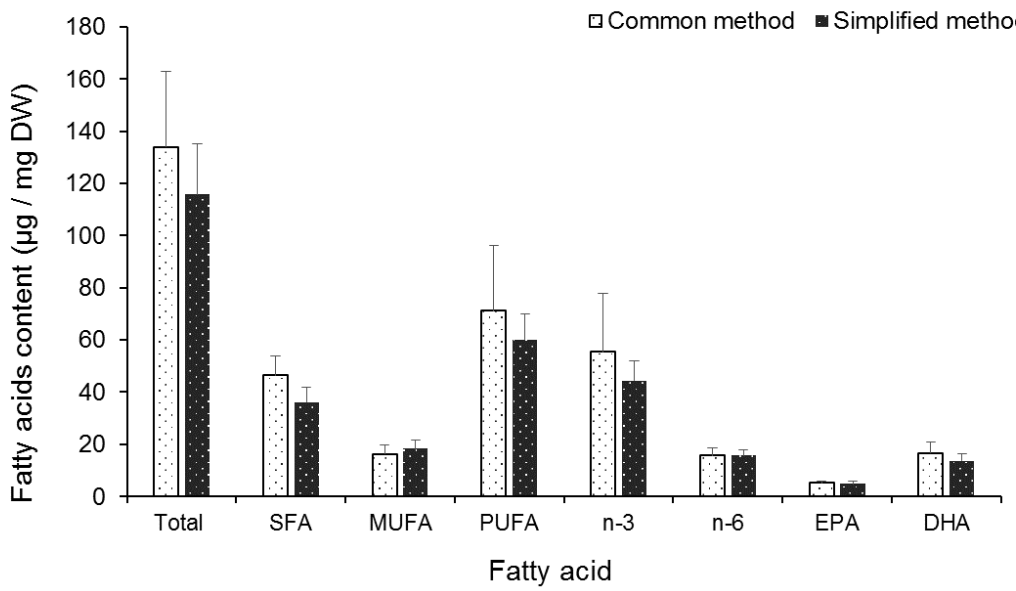

Fig. 2.2 Content of fatty acid of *A. royi* in different FAMEs preparation methods. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, n-3: omega-3 polyunsaturated fatty acids, n-6: omega-6 polyunsaturated fatty acids, EPA: eicosapentaeonic acid and DHA: docosahexaneoix acid. Data are presented as mean ± SD.

Table 2.1 Fatty acid composition (% total fatty acid) of *A. royi* in different FAMEs preparation methods. Data are presented as mean ± SD.

Fatty acid composition (%)		
Fatty acid	Common method (n=3)	Simplified method (n=3)
SFA		
C14:0	8.37 ± 1.22	7.14 ± 0.86
C15:0	0.40 ± 0.10	0.30 ± 0.02
C16:0	18.84 ± 0.86	16.86 ± 0.83
C18:0	6.38 ± 0.63	5.94 ± 0.49
C21:0	1.04 ± 0.10	1.08 ± 0.11
MUFA		
C16:1	3.87 ± 1.19	3.41 ± 0.49
C18:1	8.92 ± 3.84	11.89 ± 1.21
C20:1	nd	1.42 ± 0.09
n-6 PUFA		
C18:3 n-6	1.02 ± 0.37	1.42 ± 0.05
C18:2 n-6	9.41 ± 0.50	10.07 ± 0.64
C20:4 n-6 (ARA)	0.41 ± 0.36	1.09 ± 0.50
C22:5 n-6	0.90 ± 0.10	0.97 ± 0.13
n-3 PUFA		
C18:4 n-3	11.84 ± 0.19	12.82 ± 0.52
C18:3 n-3 (ALA)	10.88 ± 6.41	7.85 ± 0.28
C20:5 n-3 (EPA)	3.98 ± 0.44	4.24 ± 0.70
C20:4 n-3	1.36 ± 0.32	1.74 ± 0.19
C22:6 n-3 (DHA)	12.39 ± 0.75	11.75 ± 0.53

2.3.2 Salinity experiment

Population experiment-population growth and clutch size

The effects of salinity on the population growth of *A. royi* are shown in Fig. 2.3. The significantly ($p < 0.05$) largest final population was found at salinity 20 (1917.25 ± 316.5 individuals), and the smallest populations were found in both low- and high-salinity treatments (salinity 0: 67.25 ± 12.8, salinity 5: 229.25 ± 58.6, salinity 30: 237.25 ± 23, and salinity 35: 85.25 ± 38 individuals). The salinity significantly

affected the copepod composition in various developmental stages (Fig. 2.4); in particular, the highest number of nauplii occurred at salinity 20 (1400 ± 362.96 nauplii). Fig. 2.5 shows the clutch size of *A. royi* under different salinity treatments; the results were significantly-lower at salinity 0 and 5 (11.88 ± 2.37 and 16.21 ± 5.12 eggs/ clutch, respectively). However, there were no significant differences among other treatments.

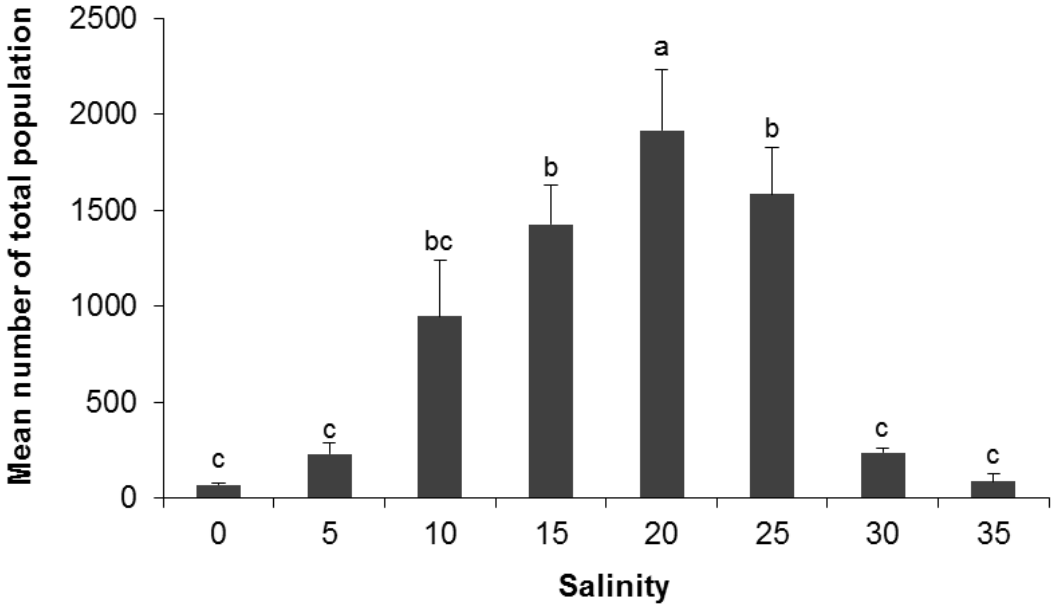

Fig. 2.3 Mean total number of final population after 14-d cultivation at the various salinity treatments. Data are averaged from 4 replicates and presented as mean ± SD. The different letters (a,b,c) above each bar indicate significant differences ($p < 0.05$).

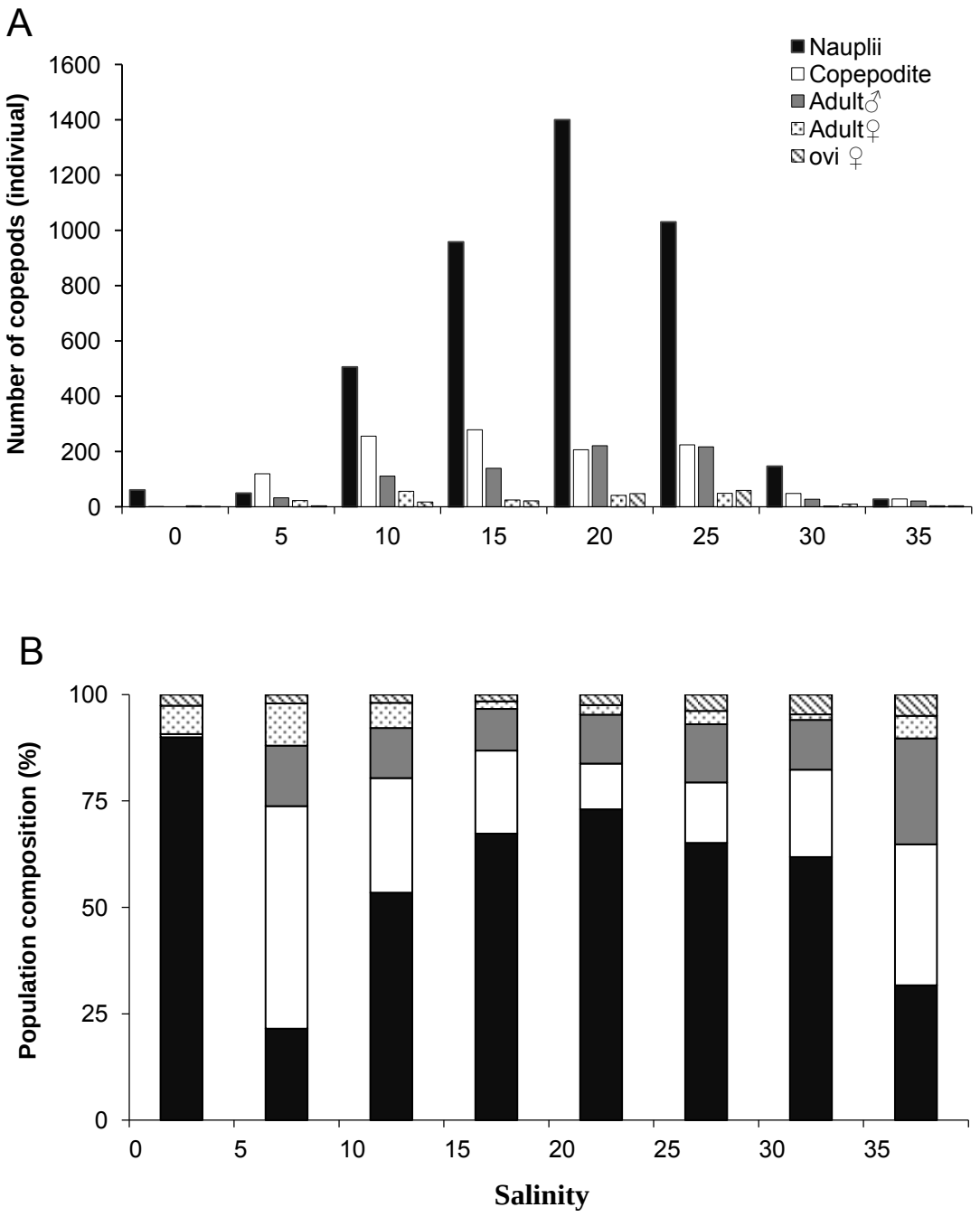

Fig. 2.4 (a) Mean number of different developmental stages (nauplii, copepododite, adult male, adult female and ovigerous female) (b) Composition (%) of different developmental stages in the cultures initiated with 8 ovigerous females at the various salinity treatments after 14 days. Data are averaged from 4 replicates.

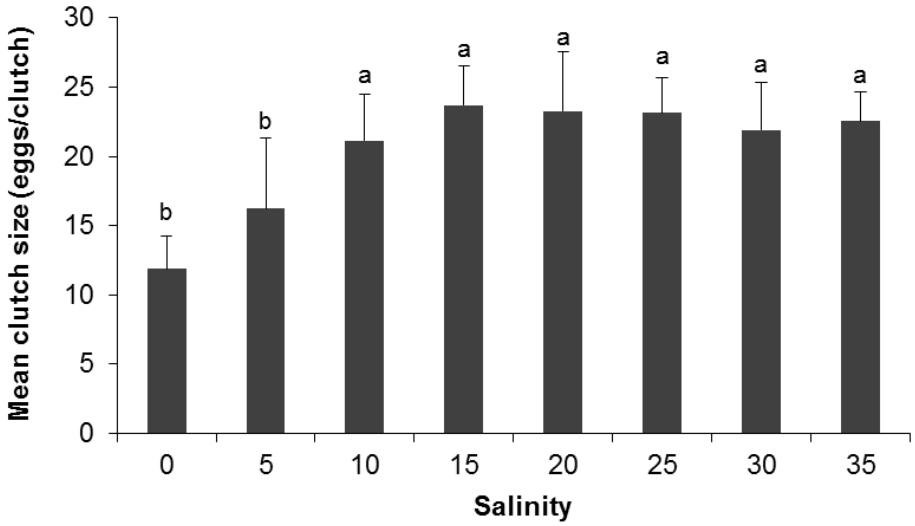

Fig. 2.5 Effects of salinities on the clutch size of *A. royi*. Data are presented as mean ± SD, and the different letters (a,b) above each bar indicate significant differences ($p < 0.05$).

Individual experiment-nauplii production and clutch production

Fig. 2.6 shows the results of daily nauplii production per female during 14 days. The significantly highest nauplii production showed at salinity 20 (12.74 ± 1.40 nauplii/ female·day) and salinity 10 (12.69 ± 1.44 nauplii/ female·day) treatments. The second highest nauplii production was salinity 30 treatment (10.25 ± 1.27 nauplii/ female·day) and the significant lowest nauplii production revealed in salinity 0 treatment (1.51 ± 0.73 nauplii/ female·day). The result of clutch production shows in Fig. 2.7, the salinity 0 treatment showed the significantly lowest clutch production during 14 days, there is no significant difference among other treatments (salinity 10, 20 and 30).

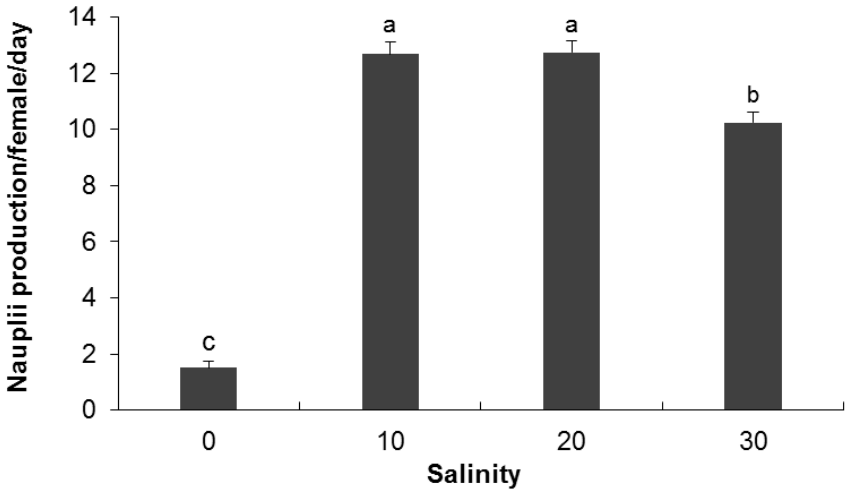

Fig. 2.6 Effects of salinities on the nauplii production per female per day during 14-d cultivation. Data are presented as mean ± SD, and different letters (a,b,c) above each bar indicate significant differences ($p < 0.05$).

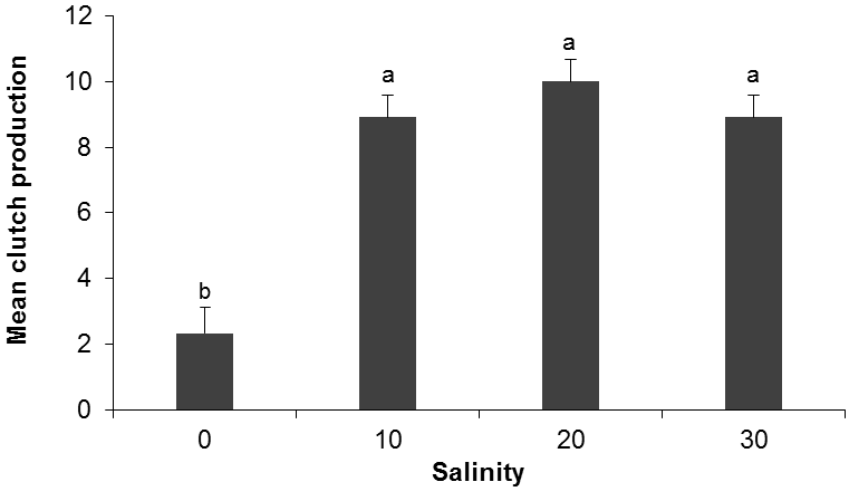

Fig. 2.7 Effects of salinities on the clutch production per female during 14-d cultivation. Data are presented as mean ± SD, and different letters (a,b) above each bar indicate significant differences ($p < 0.05$).

2.3.3 Algal diet experiment

Population growth and fecundity

The population growth and composition of *A. royi* was significantly affected by the algal diets, the result is shown in Fig. 2.8. The significant highest final population was found in ISO + NAN treatment (1911.0 ± 67.44 individuals). The second highest treatment was NAN + TET treatment (1635.3 ± 39.60 individuals), and it is not significantly different from ISO + NAN and ISO treatment (1509.3 ± 21.93 individuals). Lower results revealed in ISO + TET (1133.3 ± 246.77 individuals), TET (1089.3 ± 104.60 individuals) and NAN (914.6 ± 209.55 individuals) treatments. The significantly-lowest population was the triple species diet ISO + NAN + TET (104.0 ± 21.93 individuals). Fig. 2.9 shows the fecundity (number of eggs/ female) of *A. royi* fed on different algal diets. The highest fecundity revealed in ISO + NAN treatment (22.1 ± 0.3 eggs/ female), and it is not significantly different from NAN + TET treatment (21.4 ± 0.3 eggs/ female) and ISO + TET (20.9 ± 0.2 eggs/ female). The significantly lower fecundities were found in all single species diets: ISO (19.7 ± 0.3 eggs/ female), NAN (19.0 ± 0.2 eggs/ female) and TET (17.8 ± 0.3 eggs/ female). The result of three-species diet was excluded in this observation due to the absence of ovigerous females in the final population.

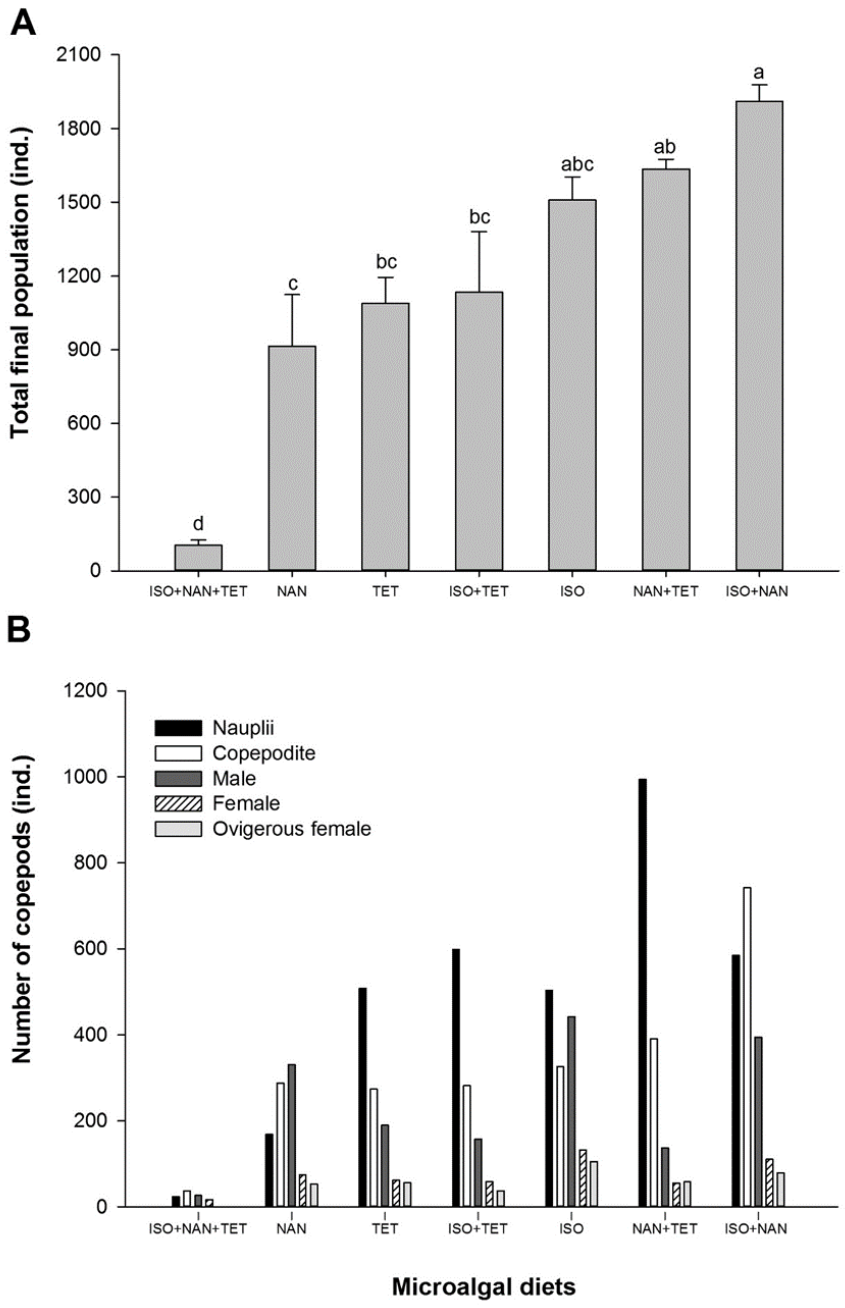

Fig. 2.8 (A) Effects of different microalgal diets on the population growth of *A. royi*. Data are presented as mean ± standard errors, and the different letters (a,b,c,d) above each bar indicate the significant differences ($p < 0.05$). (B) Mean number of *A. royi* at different developmental stages (nauplii, copepodites, adult males, adult females, and ovigerous females). ISO = *I. galbana*, NAN = *N. oculata*, TET = *T. chui*.

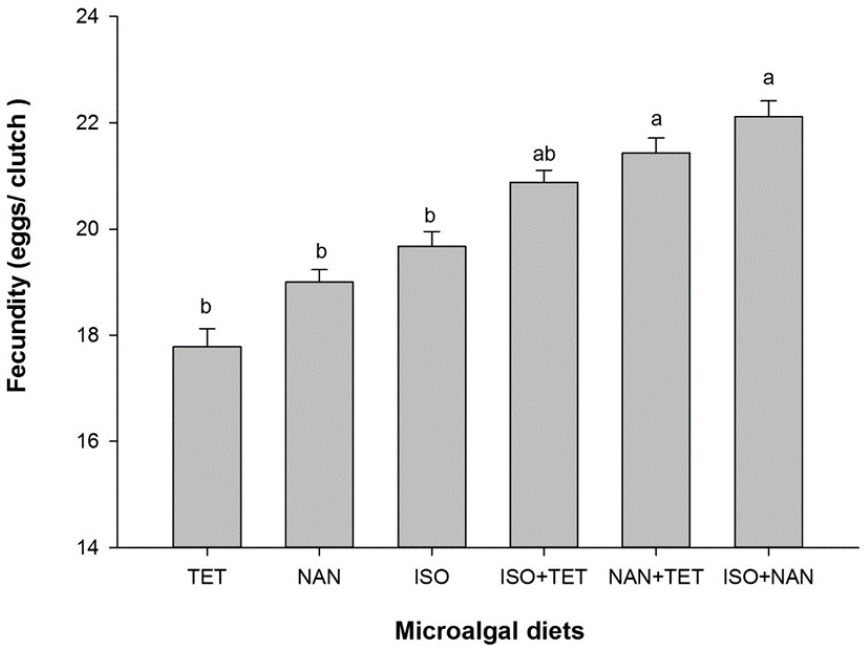

Fig. 2.9 Effects of algal diets on the fecundity of *A. royi*. Data are presented as mean ± SD, and the different letters (a,b) above each bar represents significant differences ($P < 0.05$). TET: *Tetraselmis chui*; NAN: *Nannochloropsis oculata*; ISO: *Isochrysis galbana*.

Fatty acid analysis

Fatty acid compositions varied in three species of microalgae (Table 2.2). Total n-6 polyunsaturated fatty acids (n-6 PUFA) were relatively lower (< 12%) than other groups in all microalgae. ISO and TET had a higher content of C18:2 n-6 (7.33 ± 0.13 % and 7.65 ± 0.65 %) compared to NAN (3.34 ± 0.72 %), and greater amount of C20:4 n-6 was found in NAN (4.52 ± 0.39 %) instead of ISO (not detected) and TET (0.92 ± 0.05 %). Notably, the lipid of all microalgae is dominated by n-3 PUFA fatty acids (ISO: 44.2 ± 0.82 %, NAN: 35.20 ± 2.68 % and TET: 44.48 ± 4.47%). TET showed the highest content of C18:3 n-3 (23.64 ± 3.95 %) than ISO (7.7 ± 0.66 %) and NAN (not detected). NAN had richest composition in 20:5 n-3 (35.20 ± 2.68 %) followed by TET (4.15 ± 0.27 %). Conversely, ISO had only scarce content of 20:5 n-3 (0.61 ± 0.08 %),

but a greater proportion of C18:4 n-3 (25.33 ± 0.27 %) and 22:6 n-3 (10.55 ± 0.86 %) than NAN and TET.

Fatty acid compositions of *A. royi* altered when they fed different microalgal diets (Table 2.3). Total n-6 PUFA is poorly detected (< 11%) in the fatty acids of *A. royi* where C18:2 n-6 dominated in this group by the range of 5.36 to 7.13 %. It is worth to mention that C20:4 n-6 was found in all treatments in a relatively constant proportion ranged from 1.07 to 1.82 %. The total n-3 PUFA of *A. royi* were ranged from 23.20 to 33.73 % between the various treatments. ISO treatment had the greatest content of n-3 PUFA where consisted the highest proportion of C22:6 n-3 compared to other treatments. In addition, the highest C22:6 n-3/ C20:5 n-3 ratio was also revealed in ISO treatment.

Table 2.2 Fatty acid compositions (% of total fatty acid) of three microalgae. Data are presented as mean ± standard deviation (n = 3). Abbreviations: ISO = *I. galbana*, NAN = *N. oculata*, TET = *T. chui*, nd = not detected, SFA = Saturated fatty acids, MUFA = Monounsaturated fatty acids, PUFA = Polyunsaturated fatty acids, ARA= Arachidonic acid, ALA= alpha-Linolenic acid, EPA= Eicosapentaenoic acid, DHA= Docosahexaenoic acid.

Fatty acid	Microalgae		
	ISO (3-6 μm)	NAN (2-4 μm)	TET (13-15 μm)
SFA			
C14:0	10.14 ± 1.57	4.12 ± 1.06	nd
C15:0	0.38 ± 0.03	0.23 ± 0.03	nd
C16:0	12.85 ± 0.27	24.55 ± 0.62	21.59 ± 5.51
C18:0	1.22 ± 0.88	1.66 ± 1.2	3.13 ± 0.24
MUFA			
C16:1	6.86 ± 0.94	22.57 ± 1.67	2.20 ± 0.22
C18:1	14.40 ± 0.74	3.08 ± 0.8	12.61 ± 3.73
C20:1	nd	nd	0.67 ± 0.02
n-6 PUFA			
C16:2 n-6	0.89 ± 0.1	0.25 ± 0.11	nd
C16:3 n-6	nd	nd	2.47 ± 0.29
C18:3 n-6	0.78 ± 0.06	0.49 ± 0.24	nd
C18:2 n-6	7.33 ± 0.13	3.34 ± 0.72	7.65 ± 0.65
C20:4 n-6 (ARA)	nd	4.52 ± 0.39	0.92 ± 0.05
C22:5 n-6	0.97 ± 0.1	nd	nd
n-3 PUFA			
C16:4 n3	nd	nd	13.39 ± 1.30
C18:4 n-3	25.33 ± 0.27	nd	3.30 ± 0.33
C18:3 n-3 (ALA)	7.7 ± 0.66	nd	23.64 ± 3.95
C20:5 n-3 (EPA)	0.61 ± 0.08	35.20 ± 2.68	4.15 ± 0.27
C22:6 n-3 (DHA)	10.55 ± 0.86	nd	nd
ΣSFA	24.58 ± 0.71	30.55 ± 0.65	24.22 ± 5.47
ΣMUFA	21.26 ± 0.23	25.65 ± 2.17	19.76 ± 3.87
Σn-6	9.97 ± 0.12	8.59 ± 0.97	11.04 ± 0.99
Σn-3	44.2 ± 0.82	35.20 ± 2.68	44.48 ± 4.47
Σ PUFA	54.16 ± 0.93	43.79 ± 2.33	55.52 ± 5.47

Table 2.3 Fatty acid compositions (% of total fatty acid) of *A. royi* fed different algal diets. Data are averaged from two replicates. ISO = *I. galbana*, NAN = *N. oculata*, TET = *T. chui*, nd = not detected, SFA = Saturated fatty acids, MUFA = Monounsaturated fatty acids, PUFA = Polyunsaturated fatty acids, ARA= Arachidonic acid, ALA= alpha-Linolenic acid, EPA= Eicosapentaenoic acid, DHA= Docosahexaenoic acid.

Fatty acids	Copepods fed on different algal diets						
	ISO	NAN	TET	ISO+NAN	ISO+TET	NAN+TET	ISO+NAN+TET
SFA							
C14:0	3.64	1.41	0.88	2.68	2.45	1.37	2.37
C15:0	0.38	0.38	0.52	0.50	0.23	0.41	0.38
C16:0	19.39	28.96	28.45	28.41	22.02	27.21	25.35
C18:0	11.01	9.59	5.58	8.42	5.70	8.45	7.18
C20:0	nd	1.63	0.43	0.87	0.15	0.93	0.70
C21:0	1.42	nd	nd	0.65	1.14	nd	0.22
MUFA							
C16:1	3.54	12.87	2.22	9.16	4.58	9.75	8.61
C18:1	16.30	13.56	28.73	14.15	23.22	18.58	18.03
C20:1	nd	nd	0.97	nd	0.55	0.53	0.26
n-6 PUFA							
C16:3 n-6	nd	nd	0.39	nd	nd	nd	nd
C16:2 n-6	nd	0.21	0.49	0.31	nd	nd	0.19
C18:3 n-6	0.11	nd	nd	nd	nd	nd	nd
C18:2 n-6	6.86	6.27	5.64	7.13	6.61	5.36	5.79
C20:4 n-6 (ARA)	1.34	1.28	1.36	1.07	1.26	1.82	1.36
C20:2 n-6	nd	nd	nd	0.45	nd	nd	0.41
C22:5 n-6	2.28	0.66	0.18	2.01	1.40	0.55	1.65
n-3 PUFA							
C16:4 n-3	nd	nd	3.59	nd	1.07	1.95	1.29
C16:3 n-3	nd	nd	2.89	1.07	nd	nd	0.77
C18:4 n-3	6.91	nd	1.52	2.71	4.61	1.36	2.65
C18:3 n-3 (ALA)	3.68	7.82	7.70	7.05	7.41	10.14	8.53
C20:5 n-3 (EPA)	3.12	3.38	4.09	2.63	3.37	4.14	3.56
C20:4 n-3	1.02	nd	0.33	nd	0.83	0.11	0.46
C22:6 n-3 (DHA)	18.99	11.99	4.05	10.73	13.41	7.35	10.25
ΣSFA	35.83	41.96	35.87	41.53	31.69	38.36	36.19
ΣMUFA	19.85	26.43	31.91	23.31	28.34	28.86	26.89
Σn-6	10.59	8.41	8.05	10.97	9.27	7.73	9.41
Σn-3	33.73	23.20	24.17	24.19	30.70	25.05	27.51
ΣPUFA	44.32	31.61	32.22	35.16	39.97	32.78	36.91
DHA/EPA	6.09	3.55	0.99	4.09	3.98	1.77	2.88

2.4 Discussion

2.4.1 Salinity experiment

In estuaries and brackish waters, salinity fluctuations are the crucial factor affecting the distribution of copepod populations (Cervetto et al., 1999; Lawrence et al., 2004; Devreker et al., 2009). Interspecific differences in salinity tolerance explain the variation of dominant copepod species in salinity gradients (Lance, 1963; Calliari et al., 2006). Although euryhaline species such as *Acartia tonsa* (Chinnery and Williams, 2004), *Eurytemora affinis* (Devreker et al., 2004), *Tigriopus japonicus* (Kwok & Leung, 2005), and *Pseudodiaptomus annandalei* (Chen et al., 2006; Beyrend-Dur et al., 2011) can survive under a wide range of salinities, copepod reproductive performance may change extensively depending on the salinity (Støttrup, 2000; Devreker et al., 2009). Investigating the effects of salinity on the different reproductive parameters of a copepod species could elucidate copepod population dynamics in their natural habitats or in extensive aquaculture ponds. In addition, an understanding of the optimal salinity could enhance the productivity of a copepod species in the manipulated aquaculture environments.

In this study, 8 ovigerous females were used to initiate population experiments under 8 salinity treatments. After a 14-d period of cultivation, the significantly-largest population occurred under medium salinity (20); in contrast, a remarkably low population was revealed in both low- and high-salinity treatments (0, 5, 30, and 35). In general, copepod clutch size (eggs/clutch) is a notable parameter that determines the recruitment of nauplii in a growing population (Ara, 2001). Therefore, the clutch

sizes of the ovigerous females sorted from the final population were examined for all treatments. Notably, a significantly-lower clutch size was shown only in low-salinity treatments (0 and 5). We found that clutch size did not fully correspond to population growth when *A. royi* was cultured under high-salinity conditions (30 and 35). Similarly, Ohs et al. (2010) reported that for *Pseudodiaptomus pelagicus*, the copepod clutch size may not necessarily vary with salinity; however, Ohs et al. (2010b) did find marked differences in nauplii production across various salinities. Although they did not specify a detailed mechanism for this finding, they hypothesized that nauplii production might correspond to the reduced survival rate or egg hatchability of newly-hatched nauplii under suboptimal salinity conditions. According to this hypothesis, we assumed that although the capacity of egg production was only slightly affected at salinity 30 and 35, and the reduced hatchability and naupliar survival could be the reason for the unsustainability of populations under high salinities.

The population dynamics of copepods are believed to be associated with complex biological parameters such as embryonic development, postembryonic development, survival, and the life cycle (Miller & Marcus, 1994). To obtain more comprehensive information on how salinity may affect *A. royi*, additional individual experiment was conducted. Nauplii and clutch production were observed for 12 adult pairs at 4 salinities (0, 10, 20, and 30) that were selected because of their distinguished statistical variation in the population experiments. The treatment of salinity 20 showed the significantly-highest nauplii and clutch production, indicating that salinity 20 is the optimal salinity for both individual reproduction and the population development of *A. royi*. Although a significantly-lower population growth occurred at

salinity 10, no statistical difference was detected in nauplii production, clutch size and production at the salinities of 10 and 20; a low naupliar survival rate is a possible explanation for this reproductive trend. Similar reproductive patterns were found at salinity 30, and clutch size and production were also not significantly-different from the treatment of salinity 20. However, *A. royi* had a relatively-lower nauplii production in the treatment of salinity 30 compared with the 10 and 20; this suggests that *A. royi* maintain a normal spawning ability under high salinity, but that nauplii were not produced under hyperosmotic stress. It is possible that *A. royi* has lower egg hatchability under high-salinity conditions; however, we do not have the direct data to show the hatchability of *A. royi* under various salinities because of the difficulty in estimating the initial egg numbers in egg-carrying copepods.

The evidence from the population and individual experiments suggested that the nauplii survival rate was a major parameter affecting the population growth of *A. royi* at the salinity range of 10 to 30. The naupliar stages are considered the most sensitive life stages to environmental parameters (Tester & Turner, 1990). When nauplii are exposed to adverse salinities, they may suffer from retarded development, molting failure, or even mortality (Devreker et al., 2004). Copepods at younger stages are relatively sensitive to suboptimal salinities; consequently, copepod nauplii survive in a more limited salinity range compared to adults. Devreker et al. (2004) reported that extreme salinities (i.e., 0 and 35) had a negative effect on the naupliar survival of *Eurytemora affinis*. Although adult *Eurytemora velox* was found to survive under fresh water conditions for a long time, their population was unsustainable because of the failed development of the nauplii (Nagaraj, 1988). Chinnery and Williams (2004)

revealed that the nauplii of four *Acartia* species had a higher survival rate under their preferred salinity condition (33.3).

Different reproductive patterns were found in the freshwater treatment (salinity 0). Clutch size, clutch production, and nauplii production were all significantly lower than under other salinities. Adult *A. royi* appeared to be stressed in the freshwater environment; the direct negative effect of the low osmotic condition caused a suppressive ontology of eggs in adult females. Dexter (1993) found a similar salinity preference in another *Apocyclops* species, *A. dengizicus*, which also showed greater physiological stress under low salinities. The *Apocyclops* species often dominate plankton communities in brackish coastal lagoons, saline inland lakes, and coastal salt marshes in tropical and temperate zones, and mostly occur at salinities less than 30 (Reid, 2002). Compared to open oceans and fresh waters, salinity can be extremely variable in saline inland waters; a hypersaline condition can be caused by evaporation, and hyposalinity can occur with rainfall. *Apocyclops* copepods may innately have a higher salinity tolerance to adapt to temporary salinity fluctuations in their natural habitat. However, absolute zero salinity is relatively rare in inland saline or brackish waters, which may explain why the *Apocyclops* species had a poorer physiological performance under fresh water conditions. Recently, Ibrahim et al. (2015) showed a clear, irreversible damage on the muscle structure of the euryhaline copepod *Pseudodiaptomus marinus* when exposed to salinity 0. This damage was not observed when copepods were exposed to salinity 15. This suggests that euryhaline copepods can survive in habitats characterized by high fluctuation of salinity, but only a narrow range of salinities can be optimal for their physiological processes and fitness.

A number of studies have reported the altered physiological processes of copepods in relation to unsuitable salinity conditions (Dexter, 1993; Cailleaud et al., 2007; Kaminski et al., 2014; Svetlichny & Hubareva, 2014; Peck et al., 2015). Indeed, copepods spend excessive energy on adjusting their metabolism to synthesize or degrade non-essential proteins for regulating intercellular solute concentrations when they remain under disadvantageous osmotic conditions (Kimmel & Bradley, 2001). The lower reproductive performance and individual development of *A. royi* are related to the reduced energy budget for reproductive biological processes caused by the increased energy consumption required for osmoregulation. Nevertheless, euryhaline copepods may modify their physiological processes to adapt to adverse salinities after long-term, multigenerational acclimation (Lee & Petersen, 2003). *A. royi* were shortly acclimated in 1 hour from their original salinity of 20 (the salinity measured in the pond where they were firstly collected) to the various salinities investigated in the present study. Although changing reproductive performances were revealed for the low and high salinities during the 14-day cultivation, *A. royi* may have the potential to reach their regular productivity under a variety of salinities if they are acclimated for a long period. Future studies should conduct a long-term observation to elucidate the detailed mechanism underlying salinity acclimation in *A. royi*.

This experiment determined the optimal salinity conditions for the reproduction of *A. royi*. With these results, the potential effects of salinity variations on natural copepod populations can be understood from an ecological point of view. The introduced protocol offers an accurate and promising approach for evaluating the biological response of egg-bearing copepods undergoing ecological stresses.

2.4.2 Algal diet experiment

It is viewed that copepods are selective feeders (Meunier et al., 2015; Azovsky et al., 2005; Tackx et al., 2003). The mode of food detection of copepods are species specific, and it may be triggered by various signals release from prey items such as hydromechanics, tactile stimuli and olfaction (Gonçalves et al., 2014). However, a successful ingestion is most related to the prey size suitability because the foods should be large enough to retain on the fine-meshed filtering mouthparts (the maxillae) (Boyd, 1976; Li et al., 2008). Therefore, depending on the physiological structure, prey size spectrum vary among copepod species. In this study, we selected 3 species of microalgae of sizes ranging from 2 to 15 μm (Table 2.2) to cultivate *A. royi* in the period of 14 days. The copepods fed single-species diets were all able to generate their offspring during the experimental period. Additionally, the similar food selection and size spectrum was also noted by Farhadian et al. (2008) on the congeneric copepod species *Apocyclops dengizicus*, which suggests adult *A. royi* have the capacity to ingest selected microalgae cells in this study.

Female fecundity of copepod is an important productive trait particularly used to evaluate the advantage of diets on the maternal individuals (Lacoste et al., 2001). As the reproduction of copepods has high nutritional and energetic demand, the mixture of microalgal diet can be more beneficial because of its nutritional balance (Milione & Zeng, 2007; Jobling, 2012). Similarly, our finding indicates the higher fecundity of female *A. royi* fed on two-species diets (20.78-22.12 eggs/ clutch) compared to single-species diets (17.78-19.67 eggs/ clutch). Previous studies have emphasized the fecundity of copepod is likely to link to the content of eicosapentaenoic acid (C20:5 n-3; EPA) and docosahexaenoic acid (DHA; C22:6 n-3) in their diets (Støttrup & Jensen,

1990 ; Kleppel et al., 2005). The importance of dietary DHA and EPA content to the fecundity of *A. royi* were found in ISO+NAN and ISO+TET treatment. ISO contained high ratio of DHA while TET and NAN were both rich in EPA (Table 2.2), therefore, ISO+NAN and ISO+TET provided both DHA and EPA to potentially support egg production. Nevertheless, we found also high female fecundity (21.26 eggs/ clutch) in NAN+TET which was deficient in DHA, while particularly rich in EPA and alpha-Linolenic acid (ALA). This suggests that dietary DHA level seems to play an indirect role to the fecundity of *A. royi*. Because EPA and ALA are the metabolic precursors of DHA, and it has been revealed that cyclopoid copepods are likely to have the ability to endogenously synthesize DHA from short chain fatty acids (Lee et al., 2006; Monroig et al., 2013). Therefore, due to the high content of EPA and ALA in NAN+TET, *A. royi* may utilize self-converted DHA for maintaining the remarkably higher reproductive performance. In addition, the fatty acid metabolism also involves in the synthesis of reproductive hormones in copepods (Lee et al., 2016), and may ultimately change their reproduction. Indeed, the detailed metabolic mechanism of fatty acid in the copepod has not been established yet and it will be needed to be addressed in the future.

Population growth is a multivariate process that may correlate to the changing feeding biology of copepod in different developmental stages, egg hatching success rate and the interaction between individuals, these factors determine the success of a population extension especially in a space-limited habitats such as aquaculture tanks. Evaluating the dietary effects on overall copepod population growth after a certain duration of culture is likely to offer a comprehensive information for aquaculture application. In the present work, we cultivated eight ovigerous *A. royi* for the period of 14 days. The

results demonstrated that various microalgal treatments can considerably influence the population growth and composition of different developmental stage (Fig. 2.8).

Similar to the trend observed in fecundity examination, ISO+NAN and NAN+TET showed the highest total final population among diets. Whereas, the population composition in NAN+TET were extremely dominated by nauplii, and relatively less adults occurred (Fig.2.8). The similar patterns of high nauplii and low adult component were also found in other treatments consisting of TET, this trend is disadvantageous for sustaining the population growth and indicates the developmental delay or failure of the naupliar development. In this study, TET has the largest cell among the selected microalgae species. Although TET can be ingested by female *A. royi*, it has been established that the food size for copepod optimal growth alters as a function of developmental stages (Berggreen et al., 1988; Koski et al., 2006; Saiz et al., 2014). The large cell size may create obstacle for nauplii feeding, and Farhadian et al. (2008) have stated, in the study focused on *A. dengizicuz*, the smaller cells like Nan and ISO are much preferable than TET as the diet for naupliar stages. Camus and Zeng (2010) have also noted the complete nauplii mortality when the TET was fed to the first feeding nauplii (~70 μm) of paracalanoid copepod *Bestiolina similis*, and it is linked to the inability of ingestion on the large-sized cells. Apart the concern of large cell size, the relatively lower total lipid content over dry mass has been identified in *Teterselmis* sp. (Mourente et al., 1990), and it could weaken the actual fatty acid content ingested by the copepods. Conversely, the analysis of population composition in ISO+NAN treatment showed higher component of copepodite and adult, and the total population is also the highest in various diets. As we mentioned above, the nutritional advantage of ISO+NAN can benefit to the highest female fecundity, additionally, the small cell sizes

(ISO: 3-6 μm and NAN: 2-4 μm) may offer the food accessibility throughout the developmental stages. Therefore, based on the high nauplii recruitment and successful development of early stages, ISO+NAN is the optimal diet for the overall population growth of *A. royi*.

Three-species diets did not efficiently sustain the population growth of *A. royi* in the experimental period, and no ovigerous female occurred in the cultures. It has been reported the feeding ratio between microalgae species can influence the development of copepods (Knuckey et al., 2005). As we discussed earlier TET can be less supportive to the overall population growth, the importance of ISO and NAN are then highlighted. However, with the diluted cell concentrations for each species in ISO+TET+NAN treatment, the beneficial effects provided from ISO and NAN were reduced. It may not make the three-species diet to achieve the threshold of food requirement and eventually bring on the extremely low population growth of *A. royi*.

Assessing the fatty acid profile of copepods and their diets not only help investigate the aquaculture potentials (Van der Meeren et al., 2008 ; Rayner et al., 2015), but also to understand the trophic ecology of copepods (Dalsgaard et al., 2003; El-Sabaawi et al., 2009). The capacity of lipid conversion of copepods is species-specific. Some studies has indicated that calanoid copepods are generally lack the ability to synthesize long chain fatty acids, conversely, harpacticoid and cyclopoid copepods are reported to be able to synthesize DHA when the diet is lacking it (Desvilettes et al., 1997; Monroig et al., 2013). In our study, we found DHA and EPA content in *A. royi* fed on the diets deficient in these essential fatty acids. For instance, ISO contained trace EPA (< 0.7 % in total fatty acid), but EPA (3.12 %) was detected in the copepod fed on ISO. Moreover,

NAN and TET were both deficient (not detected) in DHA, but this fatty acid was found in the copepod fed on these microalgae. This fatty acid interaction between the copepods and algae has clearly indicated that *A. royi* can bio-convert the ALA (C18:3 n-3) to EPA (C20:5 n-3) and DHA (C22:6 n-3).

The importance of dietary DHA (22:6 n-3) and EPA (20:5 n-3) has been emphasized in many studies (Rainuzzo et al., 1997; Sargent et al., 1999; Izquierdo et al., 2000; Cahu et al., 2003). These fatty acids play important roles in maintaining the membrane structure and function of visual and neural cells, thus, the dietary deficiency in DHA and EPA may cause the delayed growth, increased mortality and reduced stress tolerance of fish (Izquierdo, 1996). With a higher physiological efficiency for larvae development as well as the larger presence in fish larval tissue, the requirement of DHA is greater than EPA in most of marine fish. It has been stated that the relative proportion of DHA: EPA in larval diet is important, and the optimal ration of DHA/ EPA has been established to be 2 (Sargent et al., 1997). In our work, we found the ratio of DHA/ EPA in *A. royi* changed among different microalgal diets, and all the copepods had the DHA/ EPA ration higher than two except TET and NAN+TET. Remarkably, high DHA/ EPA ratio revealed in ISO (6.09) and ISO+NAN (4.09) treatments which were also mentioned as a very supportive diets for productivity of *A. royi*. Consequently, we recommend ISO or NAN+ISO are the suitable foods for maintaining the superior quantity and quality culture of *A. royi*. Apart the n-3 PUFA, ARA (C20:4 n-6) has been reported as an important fatty acid for fish (Koven et al., 2001; Bell & Sargent, 2003), and this fatty acid was detected in *A. royi* fed all microalgal diets (1.07-1.87 %). Although the proportional requirement of ARA for larval fish has not been clearly investigated, the

presence of ARA in *A. royi* leads this copepod species to be a great candidate for larval fish rearing.

The great suitability of *A. royi* as a live prey was confirmed in this study because of their high productivity and nutritional value for fish larvae. We recommend that ISO+NAN is the optimal diet to support the highest overall population growth and superior fatty acid profile. Our work provided the information of reproductive pattern and potential fatty acid synthesis, which contribute to the biology and aquaculture of *A. royi*.

Chapter 3. Resting eggs of the tropical calanoid copepod *Acartia bilobata*, a potential live prey for aquaculture

* Pan, Y. J. *, Souissi, A., Hwang, J. S., & Souissi, S. (2016, in press online). Artificially cold-induced quiescent egg viability of the tropical copepod *Acartia bilobata* (Copepoda, Calanoida). Aquaculture Research. DOI: 10.1111/are.12968

Pan, Y. J. *, Souissi, A., Sadovskaya I., Hwang, J. S., & Souissi, S. (in prep.) Egg dormancy of tropical copepod: egg hatching rate and fatty acid composition in egg of *Acartia bilobata* (Calanoida, Copepoda) over different cold storage durations.

Pan, Y. J. *, Souissi, A., Hwang, J. S., & Souissi, S. (in prep.) Photoperiodic effects on the egg and diapause egg productions of the tropical copepod *Acartia bilobata* (Calanoida, Copepoda).

3.1 Literature review

Copepods are dominant in plankton communities, as an essential trophic linkage, copepods transfer photosynthesized energy from primary producer to higher-trophic-level consumers such as fish (Turner, 2004). The abundance of copepod in the water column is undoubtedly one of the key factors correlated with the population recruitment of fish (Beaugrand et al., 2003). Naupliar copepods, in particular, are the major developmental stages that are preferably selected by fish larvae as foods (Turner, 1984; Sampey et al., 2007). Therefore, the recruitment of nauplii is either critical for maintaining copepod population and for sustaining the fish population in the wild. Copepod subitaneous eggs and resting eggs are two possible resources for nauplii recruitment (Glippa et al., 2014). The majority of nauplii hatch rapidly (e.g. few days) from subitaneous eggs, and it may directly contribute to the increase of present copepod population. Another portion of nauplii originate from resting eggs, such eggs in fact form an 'egg bank' in the sediments of aquatic environments (Hairston et al., 1995; Hairston, 1996; Brendonck & De Meester, 2003; Glippa et al., 2011), and it is a critical ecological strategy regulating copepod population over seasonal variation (Uye, 1985; Marcus, 1996).

To date, resting egg has been observed in three copepod orders: Calanoida, Harpacticoida, and Cyclopoida (Dahms, 1995). Although the detailed biological mechanisms have not been well established in resting copepod eggs, two major groups have been classified (Uye, 1985). The first group refers to egg dormancy, which is sated in a status of arrested development, and those eggs need to experience refractory phase until the resume of embryonic development. The dormant eggs normally occur when the maternal individuals receive the signals of deteriorating

conditions such as unfavorable photoperiod and temperature regime (Piercey & Maly, 2000). Two types of egg were further identified in this group, including diapausing and delayed-hatching egg (Drillet, 2010). Diapausing egg take relatively longer time to resume from the refractory phase even when the conditions turn to be suitable (Grice & Marcus, 1981). Delayed-hatching egg is reported as an intermediate dormant type which has a shorter refractory phase than the diapause eggs (Chen & Marcus, 1997). The second group is quiescence, which develops when the subitaneous egg is exposed to a sudden suboptimal condition. Quiescent eggs maintain sluggish metabolism in embryo and are able to hatch rapidly once the environmental conditions become favorable again (Marcus, 1996).

Laboratorial studies have been carried out to investigate the possible trigger signals that metabolically decelerate the development of copepod egg by artificial stimulations. As the development of copepods are temperature-depending (Huntley & Mai, 1992), the technique of cold storage on egg have been recently addressed on the wide-disturbed calanoid species *Acartia tonsa* (Støttrup et al., 1999; Holmstrup et al., 2006; Drillet et al., 2006a; Drillet et al., 2007). Drillet et al. (2006a) reported the viability in the egg of *A. tonsa* (Baltic population) after 12-month cold storage at 2-3 °C, and he stated that the cold storage method extends the embryonic development time and switches the eggs of *A. tonsa* into quiescence. Apart the observations on the hatching characteristic, chemical composition of copepod eggs provide the cues to explore the metabolic progresses in copepod eggs as well as their nutritional value as food for fish larvae over cold storage times. The change of fatty acids level, however, in cold-stored eggs of *A. tonsa* seems to be minor (Støttrup et al., 1999; Drillet et al., 2006a; Sedlacek, 2008), and this finding may highlight the trophic significance of the

nauplii derivate from resting eggs in the marine food web and potential to be used as live food for larval fish in aquaculture. On the other hand, photoperiod is one of the significant seasonal signals, which regulates the variation of copepod population in their natural habitat. At copepod culture facilities, it could be a key manipulative factor controlling the productivity of copepods in low cost (Camus & Zeng, 2008). Some reports have revealed that photoperiod can affect morphology and reproduction of copepods (Matias-Peralta et al., 2005; Camus & Zeng, 2008). Most interestingly, the photoperiod have been also reported to influence the production and hatching of copepod diapause egg (Marcus, 1996).

Similar to the concept of brine shrimp cyst, the dormant egg of copepod aim to produce a conservable product and obtain the nauplii by incubating the eggs in the desired period for larvae production in hatchery, either for maintaining the continuous copepod culture line with the well-designed operation schedule. Production of quiescent egg is particularly interesting for aquaculture because of their immediate dormancy response and relatively rapid hatchability. To achieve the exploitability of quiescent copepod egg, the artificial induction processes have been tested with various manners, such as salinity, temperature and food limitation (Støttrup et al., 1999; Holmstrup et al., 2006; Drillet, 2010; Hagemann, 2011). However, the protocols of quiescent egg induction are mostly developed with the copepod *A. tonsa* in temperate region and no trail has been so far conducted with tropical stenothermal copepod species.

The tropical calanoid species *Acartia bilobata* is abundant in the brackish aquaculture pond and estuarine areas in southern Taiwan. Beyrend-Dur et al. (2014) described for the first time the occurrence of *A. bilobata* nauplii after few-days incubation of

long-term stored sediment samples collected from aquaculture ponds in Tungkang, southern Taiwan. This finding suggests that *A. bilobata* have high potential in producing dormant eggs for aquaculture purposes. In the present study we aimed to test the feasibility of cold-induced quiescent eggs from a laboratory cultured tropical copepod species *A. bilobata* and to examine the viability of these eggs after different cold storage duration.

In this chapter, we aimed to test the feasibility of cold-induced resting eggs from two laboratorial culture strains of tropical copepod species *A. bilobata*. In addition, we also investigated the effects of photoperiod on the egg production, egg hatching rate, population survival rate and diapause production rate of *A. bilobata.*

3.2 Material and Method

3.2.1 Copepod and microalgae stock culture

Two different strains of copepod *A. bilobata* were tested in this study. In the first trial, the long-term acclimated strain (since year 2000) was obtained from Tungkang Biotechnology Research Center, Taiwan. In the second trial, the new strain was isolated from an aquaculture pond (year 2014). The starter culture of microalgae *Isochrysis galbana* (Tahitian strain, T-ISO, Haptophyceae) was obtained from the Roscoff Culture Collection (Roscoff, France). The stable culture lines including microalgae, new and old strains of *A. bilobata* were established in LOG-Marine station of Wimereux, France. The copepods were cultivated in 20L tanks with diluted seawater at salinity 20 (mixture of distilled water and 1μm-filtered ultraviolet-sterilized seawater). The algae T-ISO was introduced every 2 day at the approximately cell concentration of 10^5 cells/ ml. The

cultures were maintained at a photoperiod of 12L: 12D, and the culture water was heated by a water heater (EHEIM thermocontrol 50 w, EHEIM, Germany) at 28 °C. Batch cultures of T-ISO were performed in 2-L flasks contained Whatman GF/C-filtrated and autoclaved natural seawater supplied with Walne's medium (Walne, 1970). Algal cultures were kept in the thermostatic incubator (MLR-351H; SANYO, Osaka, Japan) programmed at 28 °C and in a photoperiod of 12 h: 12 h light: dark cycle. The algae used for feeding copepod was in exponential growth phase (3-4 days after inoculation).

3.2.2 First trial (long-term acclimated culture strain)

About 1000 adults were transferred from the stock culture to a 2-L beaker filled with new culture water and placed in a heated water bath at 28 °C under a photoperiod of 12/12h, L/D for 24 h in order to obtain newly-spawned eggs. After spawning, the eggs were collected by sieving the culture water through 150-and 38-μm meshes. Approximately 1000~1500 eggs were placed in a 50-ml bottle filled with GF/C filtered diluted seawater (salinity 20). The egg stocks were then kept in a refrigerator at 4 °C for different storage durations (7, 14, 21 and 30 d).

After different periods of cold storage, the eggs were sorted from the bottle and counted under the stereomicroscope (SZX9, Olympus, Japan). One hundred to 150 eggs were incubated in a 25-ml dish filled with diluted seawater (salinity 20). Four replications were tested for each of the two incubation periods (48h and 72h). All dishes were incubated in a heated water bath at 28 °C under a photoperiod of 12h: 12h/ light: dark. At the end of the incubation, the number of unhatched eggs was counted and the egg hatching success rate (HSR) was calculated as follows:

$$\text{Egg hatching success rate} = 1 - \frac{\text{Number of unhatched eggs}}{\text{Number of total incubated eggs}}$$

3.2.3 Second trial (newly established culture strain)

Hatching success rate (HSR) of overall eggs after cold storage

Adult *A. bilobata* were collected by 120-μm mesh from the stock cultures, and then transferred to new 20-L cultures in order to obtain fresh-spawn eggs. Copepod eggs were siphoned from the bottoms, and rinsed through 120- and 70-μm meshes. Approximately 15000 eggs were placed in each the 15-mL brown-colored tube filled with diluted seawater (salinity 20). The egg stocks were then kept in a refrigerator at 4°C for different storage durations (7d, 14d, 30d, 60d, 90d, 120d and 365d). After different durations of cold storage, the eggs were sorted from the tubes and counted under the stereomicroscope (SZX9; Olympus, Tokyo, Japan). One hundred to 150 eggs were incubated in a 25-mL dish filled with diluted seawater (salinity 20). Four replications were tested for each of the two incubation periods (48h and 72h). All dishes were incubated in a heated water bath at 28°C under a photoperiod of 12-h light: 12-h dark. At the end of the incubation, the number of unhatched eggs was counted and the hatching success rate (HSR) was calculated following the equation mentioned in the section 3.2.2.

Fatty acid analysis

Fresh and cold-stored eggs (7d, 14d, 30d and 60d) of *A. bilobata* were sampled volumetrically and captured on Whatman GF/C filter papers by the vacuum-filter

(5000 eggs/ replicate, n = 2). The filter paper containing eggs was lyophilized, and immerse with 5 ml lipid extraction solvent (chloroform: methanol = 2: 1, v: v) added 100 μl of C17:0 internal standard (0.2mg/ ml) in 10-ml glass vial over 36 h at -20 ◦C. The sample was then sonicated at an ice bath for 2 h. Solvent was transferred in the new vial and evaporated by a gentle nitrogen stream until approximately 1 ml, then the extraction was centrifuged in an eppendorf at 13,000 rpm for 3 min. The supernatant was transferred and evaporated completely in a new 10-ml glass vials. A 2-ml transesterification reagent (methanol: toluene: acetyl chloride = 85: 66: 15, v: v) was added into the vial, the glass vial was caped and heated at 95 °C for 2h. After transesterification, the solvent was evaporated completely at 60-70 °C by the nitrogen stream. Fatty acid methyl esters (FAMEs) were solubilized in hexane (1 ml), washed with distilled water (1 ml), and the organic layer was filtered through a cotton-plugged Pasteur pipettes to a glass GC vial. The FAMEs were evaporated by a nitrogen stream until about 100 μl and conserved at -20 °C until GC-MS injection. GC–MS analysis was performed on a Trace GC ULTRA system (Thermo Scientific) equipped with a capillary column NMTR-5MS (30 m × 0.25 mm) using a temperature gradient of 170 °C (3 min) → 250◦C at 5 °C / min and with a DSQ II MS detector.

Effects of cold storage on the HSR of diapause eggs

The unhatched eggs occurred after 72-h incubation in the fresh egg treatment, and these eggs stayed unhatched and morphologically complete during an extended 5-d incubation. Such eggs, thus are defined as diapause eggs of *A. bilobata*, and the additional experiment was carried out to investigate if the chilling treatment (4°C) could trigger their hatching. Around 5,000 fresh-spawned eggs were incubated in each 2 L beaker, the unhatched eggs were siphoned from the bottom of the beakers after 72

h. These eggs were then stored at 4 °C for different storage durations (7d, 14d, 30d, 60d), and the HSR was examined as described previously in 3.2.2.

Estimation of the HSR of quiescent egg

In order to estimate the HSR of the quiescent eggs, the contribution of diapause HSR was subtracted from the overall HSR, and it was calculated as follows,

$$\text{Estimated HSR}_q = \text{HSR}_o - \text{HSR}_{dc}$$

where the HSR*q* is the estimated HSR of the quiescent eggs, the HSR*o* is the HSR of the overall eggs (refer to the data showed in Fig.3.3) and the HSR*dc* represents the contribution of HSR from the diapausing eggs, which was estimated as the unhatched rate in the fresh-spawning egg (non-cold-stored) multiplied by the HSR of diapausing eggs (refer to the data showed in Fig. 3.5).

3.2.4 Photoperiod experiment

Egg production

Five treatments (24L: 0D, 16L: 8D, 12L: 12D, 8L: 16D and 0L: 24D) were designed to investigate the effects of photoperiod on *A. bilobata*. Before the experiment started, the copepods were acclimated to various photoperiods. Around 250 adult copepods were collected from stock culture and transferred to a 2-L beaker. Three beakers were prepared for each treatment, and the cultures were kept in the thermostatic incubators

(MTI-03, Firstek Scientific, Taipei, Taiwan) programmed at 28 °C and designed photoperiods. After 10 days, 15 pairs of adult copepods were randomly sorted from each photoperiod treatment and placed respectively in the 6-well culture plates containing fresh culture medium, respectively (salinity 20, fed *I. galbana* at approx. 10^5 cells/ml). The pairs were observed every day under stereomicroscope (OLYMPUS SZX16, Tokyo, Japan), egg productions of these pairs were documented in 5 consecutive days. After each observation, the pairs were transferred in the new culture wells and the eggs were collected for the experiment of egg hatching success rate.

48-h egg hatching success rate, diapause rate and population survival

The eggs collected from the pairs were equally placed in the petri dishes containing fresh culture medium. These eggs were incubated in the thermostatic incubators which were programmed in the same photoperiodic condition of their parents. After 48 h, the number of unhatched eggs was counted under stereomicroscope, and the 48-h egg hatching success rate was calculated as:

$$\text{48h egg hatching success rate (\%)} = 100\% \times \left(1 - \frac{\text{Number of unhatched eggs}}{\text{Number of total incubated eggs}}\right)$$

After counting, the eggs were kept incubated in the thermostatic incubators, the cultures were fixed by a few drops of buffered formalin solution on the 7th day. The unhatched eggs observed in 7th day are defined as diapause eggs, the ratio of diapause egg was calculated as:

$$\text{Diapause ratio (\%)} = 100\% \times \left(1 - \frac{\text{Number of unhatched eggs on 7th day}}{\text{Number of total incubated eggs}}\right)$$

And the population survival rate was calculated as:

$$\text{Survival rate (\%)} = 100\% \times \left(1 - \frac{\text{Number of copepods on 7th day}}{\text{Number of total incubated eggs}}\right)$$

3.2.5 Data analysis

All statistical analyses of this study were run using SPSS program (SPSS, Chicago, IL, USA). The effects of different cold storage periods and photoperiods were analyzed using one-way ANOVA to compare the mean values of the desired traits. Since significant differences were detected in all treatments ($p < 0.05$); Tukey's multiple comparison test was then used to analyze specific differences between pairs of treatments.

3.3 Results

3.3.1 First trial (long-term acclimated culture strain)

Fig. 3.1 shows the effects of cold-storage duration on the egg hatching success. A significantly highest hatching success rate was found in the 7-d storage treatment after 72-h incubation (89.94 ± 2.73%) which was not significantly different compared to the 48-h incubation (89.37± 1.63%). The 14-d, 21-d and 30-d storage treatments showed a significantly lower hatching success rate than the 7-d storage treatment. However, over 50% of hatching success still occurred after 30-d of cold storage. Fig. 3.2 shows the relationship between egg hatching success rate and cold storage duration.

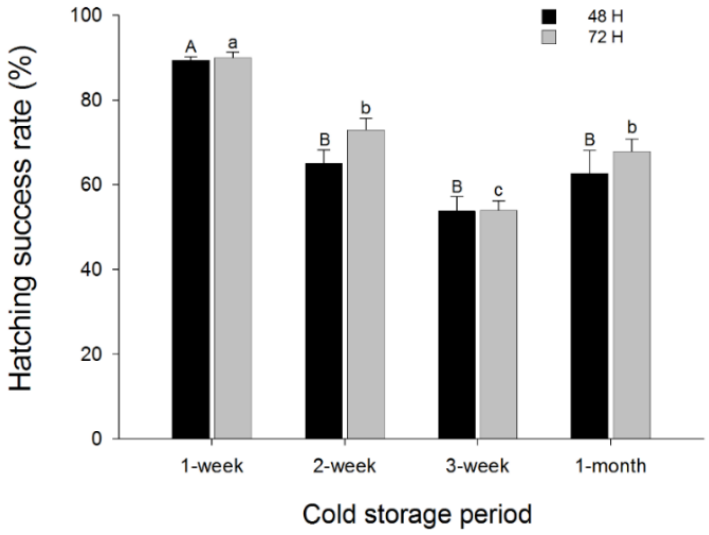

Fig. 3.1 Effects of different cold-storage durations on the hatching success rate (HSR) of *A. bilobata* in first trial (long-term acclimated culture strain). Data are presented as mean ± standard errors, and the different letters (A,B; a,b,c) above each bar represents significant differences ($p < 0.05$).

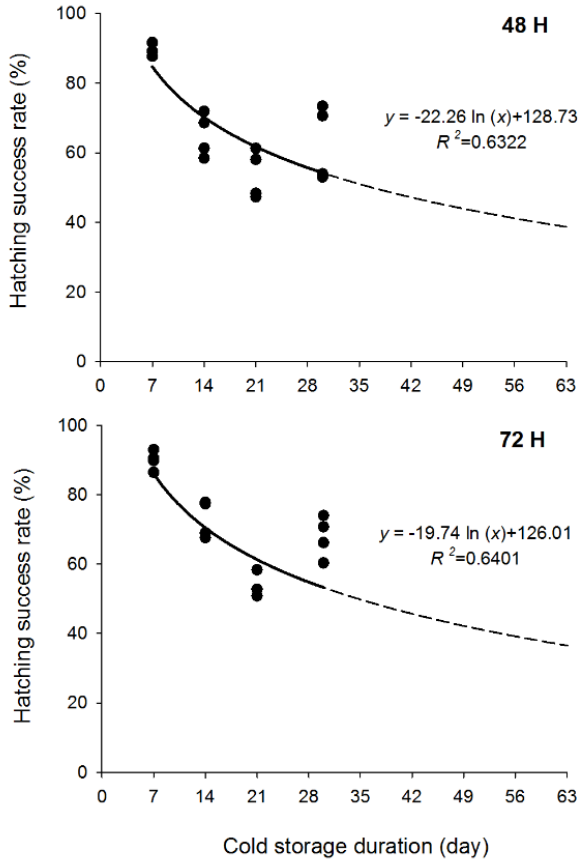

Fig. 3.2 Relationship between HSR and cold storage duration in first trial (long-term acclimated culture strain).

3.3.2 Second trial (newly established culture strain)

HSR of newly-spawned eggs after cold storage

Fig. 3.3 shows the 48-h and 72-h HSR of overall fresh-spawned eggs of *A. bilobata* after different storage durations up to 365 d. The 48-h and 72-h HSR of the fresh eggs were 66.54 ± 5.10% and 66.33 ± 2.52%. Surprisingly, the increased HSR occurred during the first two weeks of cold storage, and significantly-highest HSR were found in the 14-d storage treatment (48h: 85.82 ± 3.25%, 72h: 87.59 ± 1.78%). After the 21-d cold storage, the HSR started to decrease gradually and the significantly-lowest HSR revealed in the 365-d storage treatment (48h: 7.04 ± 1.21%, 72h: 19.44 ± 7.72%).

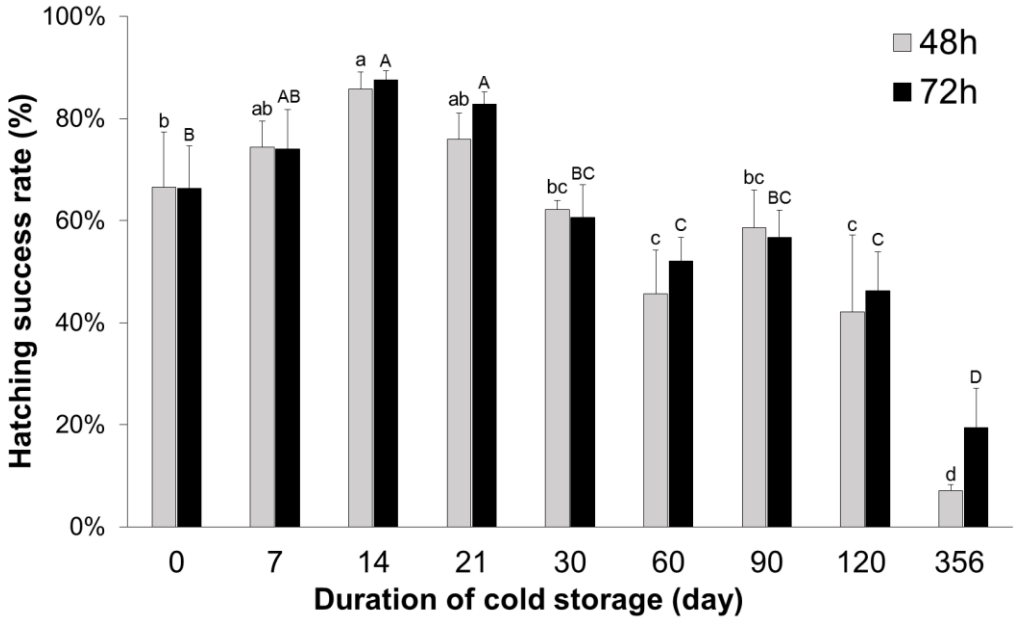

Fig. 3.3 HSR of cold-stored eggs of *A. bilobata* after different durations in the second trial (newly established culture strain). Data are presented as mean ± SD, and the different letters (A, B, C; a, b, c) above each bar represents significant differences ($P < 0.05$).

Fatty acid analysis

Fatty acid compositions of the egg of *A. bilobata* over different cold storage durations were shown in Table 3.1. Saturated fatty acids that consisted of C14:0, C15:0, C16:0 and C18:0 were dominant in the lipid of egg of *A. bilobata* in all storage durations. However, the remarkable change in the fatty acid composition showed in 14-d treatment, and these eggs had declined monounsaturated (MUFA) and undetectable polyunsaturated fatty acids (PUFA) content. Fig.3.4 illustrates the relationship between DHA composition (% total fatty acid) and the HSR of cold-stored eggs of *A. bilobata*.

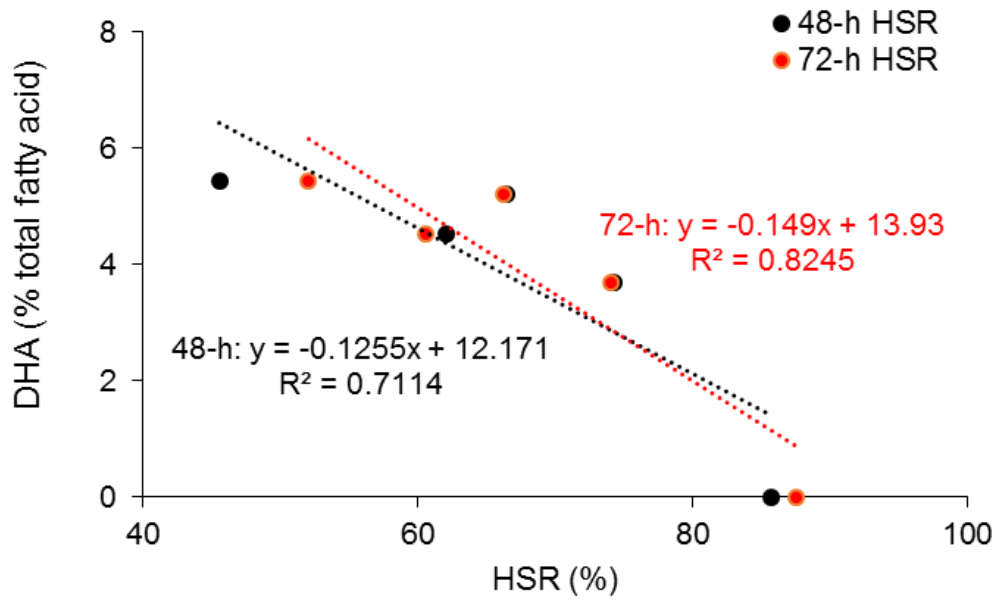

Fig. 3.4 Relationship between DHA composition (% total fatty acid) and the HSR of cold-stored eggs of *A. bilobata*.

Table 3.1 Fatty acid compositions (%) in the egg of *A. bilobata* over different cold storage durations in second trial (newly established culture strain). Data were averaged from two replicates. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, DHA: Docosahexaenoic acid.

Fatty acid	Fresh	7d	14d	30d	60d
C14:0	3.35	3.15	2.91	1.24	3.62
C15:0	1.86	1.23	1.54	0.54	1.55
C16:0	37.76	42.02	41.55	37.56	40.21
C18:0	36.45	37.58	48.47	43.73	38.69
Total SFA	79.42	83.98	94.48	83.06	84.07
C16:1	2.47	2.12	0.00	0.00	0.00
C18:1	9.60	9.09	5.52	10.98	10.49
Total MUFA	12.07	11.21	5.52	10.98	10.49
C18:2 n-6	3.29	1.11	0.00	1.44	0.00
C22:6 n-3 (DHA)	5.22	3.70	0.00	4.52	5.44
Total PUFA	8.50	4.81	0.00	5.96	5.44

Effects of cold storage on the HSR of diapause eggs

The diapause eggs turned to be viable within 72-h incubation after cold storage treatment, the 48-h and 72-h HSR after different storage durations are illustrated in Fig. 3.5. The 48-h and 72-h HSR of 7-d cold-stored diapause eggs were 63.45 ± 5.90 % and 64.07 ± 6.09 %, respectively. Similar to the hatching trend discovered in the cold-stored fresh-spawned eggs, the HSR peaked in 14-d treatment (48h: 75.28 ± 6.94 % ; 72h: 78.22 ± 4.14 %) and declined gradually during the cold storage duration up to 60 d. Fig. 3.6 illustrates the relationship of estimated HSR of quiescent eggs to different durations of cold storage.

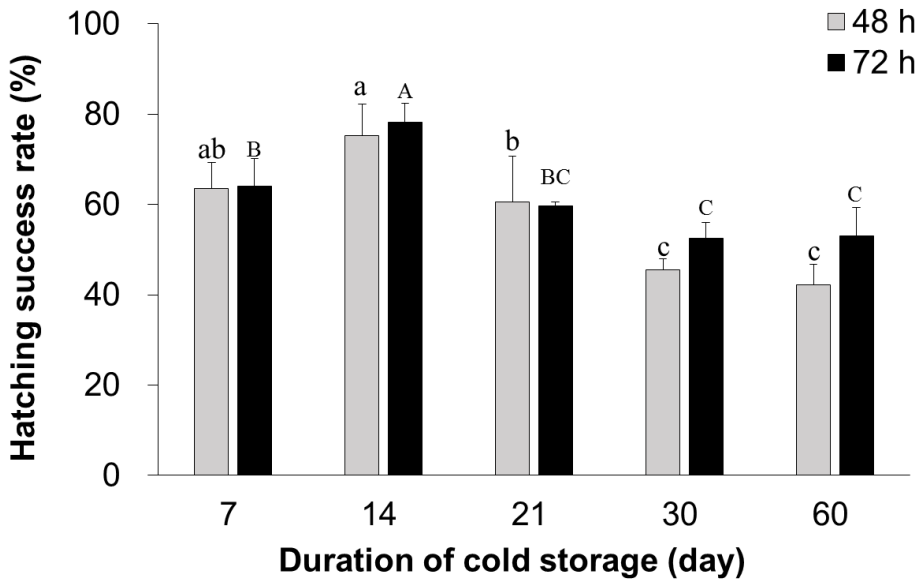

Fig. 3.5 HSR of diapause eggs of *A. bilobata* after different durations of cold storage in the second trial (newly established culture strain). Data are presented as mean ± SD, and the different letters (A, B, C; a, b, c) above each bar represents significant differences ($P < 0.05$).

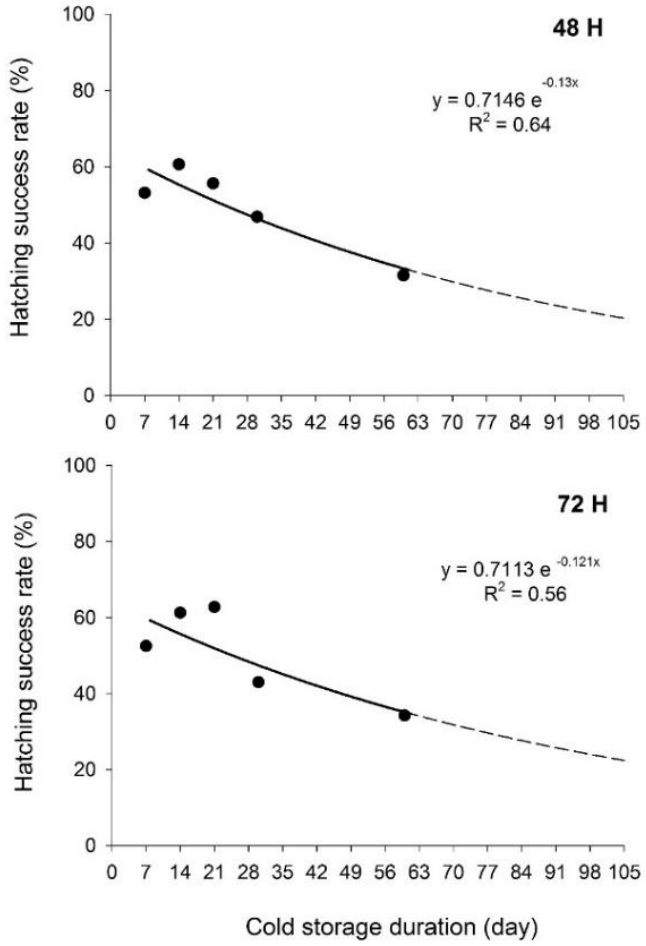

Fig. 3.6 Estimated HSR of quiescent eggs of *A. bilobata* after different durations of cold storage in the second trial (newly established culture strain). Data were estimated by subtracting the mean value of diapause HSR from the HSR of overall eggs, and plot graph was described with exponential decline fitting model.

3.3.3 Photoperiod experiment

Egg production

The daily egg production is shown in Fig. 3.7. The photoperiod significantly affected the egg production of *A. bilobata*. Highest daily egg production per female was found in 8L: 16D treatment (21.5 ± 3.3 eggs), however, which is not statistically different from 12L: 12D (16.9 ± 3.2eggs) and 16L: 8D (19.9 ± 2.8 eggs) treatments. The significantly lower egg productions were found in 0L: 24D (10.3 ± 3.3 eggs) and 24L: 0D (4.1 ± 2.5 eggs).

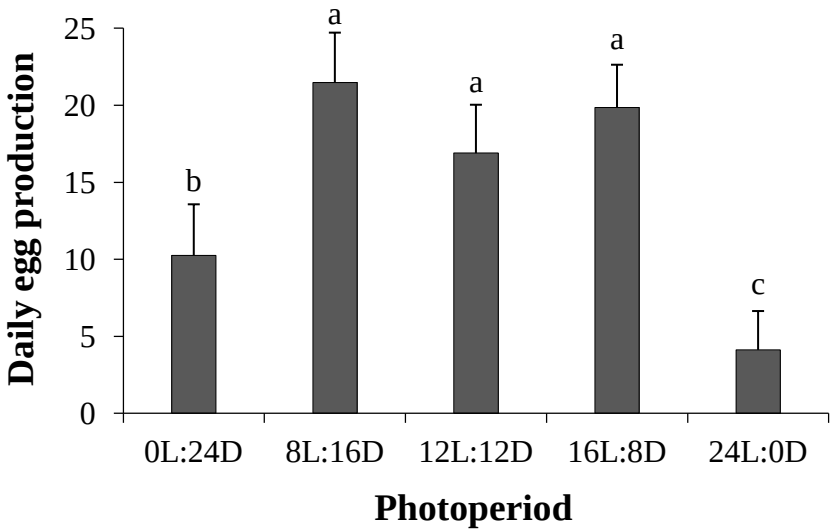

Fig. 3.7 Effects of photoperiods on the daily egg production of *A. bilobata.* Data are averaged from the egg production of 15 pairs in 5 consecutive days and presented as mean ± SD. The different letters (a,b,c) above each bar indicate significant differences ($p < 0.05$).

The 48-h hatching success rates of *A. bilobata* eggs collected from each photoperiod treatment are shown in Fig 3.8. The hatching success rates are in the range of 79-94 %, nevertheless, there was no significant difference detected among 5 photoperiods. The

survival rate of final population on 7th day is shown in Fig. 3.9. The highest population survival was found in 12L: 12D treatment though there is no statistic difference identified in 5 treatments. Fig. 3.10 shows the effects of photoperiods on the diapause egg production rate of *A. bilobata*, the significantly-lower diapause production occurred in the full-dark treatment.

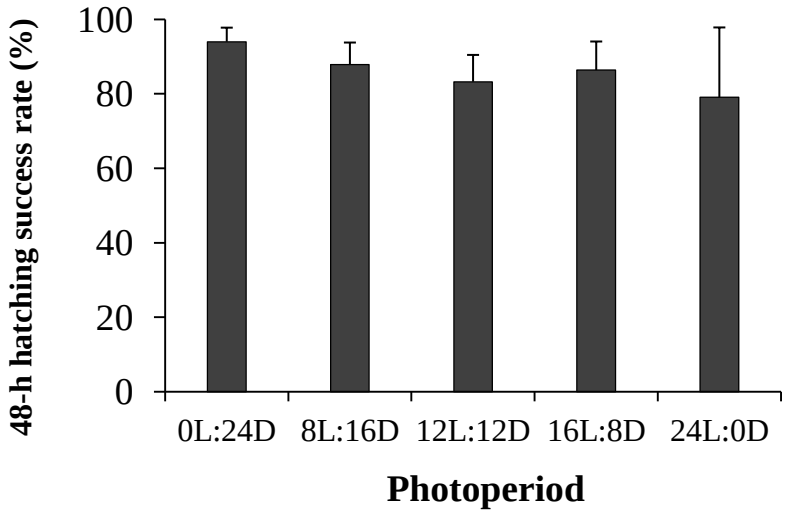

Fig. 3.8 Effects of photoperiods on the 48-h HSR of *A. bilobata*. Data are presented as mean ± SD.

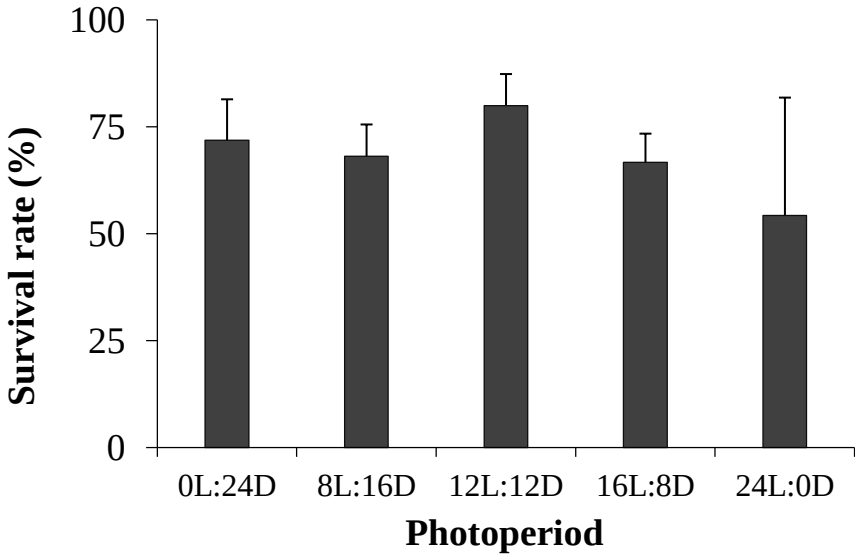

Fig. 3.9 Effects of photoperiods on the population survival of *A. bilobata*. Data are presented as mean ± SD.

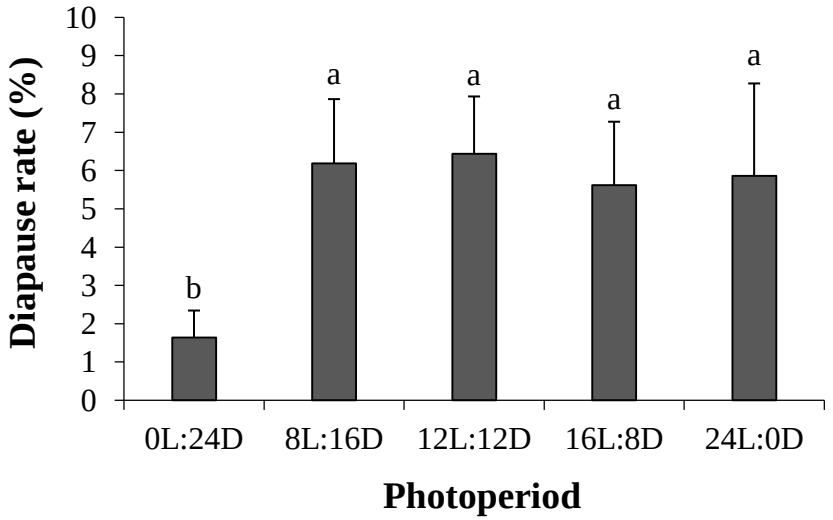

Fig. 3.10 Effects of photoperiods on the diapause egg production rate of *A. bilobata*. Data are presented as mean ± standard error. The letter (a,b) above each bar indicate significant differences ($p < 0.05$).

3.4 Discussion

3.4.1 First trial (long-term acclimated culture strain)

According to our previous study (my master thesis), we found 89% of hatching success rate of the newly-spawned eggs produced by the same strain of *A. bilobata* reared in the same conditions as in the present study (Pan et al., 2014). A similar hatching success rate of 7-d cold stored eggs and newly-spawned egg suggests that the cold environment could trigger most of the eggs to switch to a quiescent state. The rest of unhatched eggs (about 10 %) could be a failure of fertilization or a natural ratio of diapause production, indeed, the identification of different egg types should be studied in deep in the future. Since the embryo still keeps some metabolic activities in the eggs, energy is slowly consumed during cold-induced quiescence (Nielsen et al., 2006). The hatching success rate of quiescent eggs could gradually decrease with declining energy reservation and to a point where they are even unable to hatch after long-term storage (Drillet et al.,

2006a). Based on the gradual energy consumption during cold storage, the relative equations (Fig. 3.2) were released to predict the hatching rate on the *A. bilobata* egg during a longer storage period. The extrapolation of the fitted trends confirmed that after two months a relatively good hatching success around 40-45 % could be obtained. However, the viability of quiescent eggs during longer cold storage periods will be investigated in more detail in the future. This experiment summarizes the first time viability of artificially cold-induced quiescent eggs for the tropical copepod *A. bilobata*. Our findings indicated a great potential to develop quiescence eggs of *A. bilobata* for aquaculture uses in Taiwan and other tropical countries.

On the other hand, the cold storage duration is not the only factor affecting the hatchability of quiescence eggs. The hypothesis of "strain effect" was raised when very different hatching success rates were found between the cold-stored eggs of *A. tonsa* from Denmark (>25-year-old strain) and Florida, USA (2-year-old strain) (Drillet et al., 2007). The genetic differentiation between copepod strains seems to be an important parameter that influences the hatchability of both newly-spawned and cold-stored eggs (Drillet et al., 2008). In our study, the strain of *A. bilobata* had been established for more than 10 years when the experiment was conducted. To investigate the viability of eggs produced by different strains of *A. bilobata*, another experiment was carried out.

3.4.2 Second trial (newly established culture strain)

In the second trial, we found the low HSR (60-70 %) in the fresh-spawned eggs of the newly-established *A. bilobata* culture. The eggs stay unhatched and not decomposed during an additional 5-d incubation. We, therefore, considered those eggs are in their

refractory phase of dormancy. In addition, the unexpected increase (15-20 %) revealed in the HSR of 7-d and 14-d cold storage treatments indicated that part of those unhatched eggs turned to be viable after the change of temperature (28°C→4°C→28°C). Copepod diapause eggs are noted to terminate their refractory phase after a chilling or warming stimulation, whereas the hatching of delayed-hatching egg do not require the temperature change to occur (Chen & Marcus, 1997; Glippa et al., 2011). According to this definition, here we classify the unhatched eggs observed in the incubation of fresh-spawned eggs as diapause. Although it has been revealed that the structure of egg shell are different in diapause and subitaneous eggs in some copepod species (Onoue et al., 2004), we did not found the morphological difference in the eggs during our preliminary microscopy observation and a further scanning electric microscopy examination is held in the future study.

In comparison to our previous investigations, the HSR of fresh-spawned egg was lower in the newly-established culture strain of *A. bilobata* (table 3.2). This finding indicated that different copepod strains produce various ratios of egg type even when the cultures were maintained in the same condition. Genetic differentiation has been observed in different copepod populations of the same species, and it could be a multivariable mechanism. First, geographical segmentation is likely to make each copepod population develop the biological features which adapt to their habitats. For instance, a very different HSRs were found between the cold-stored eggs of *A. tonsa* from Baltic Sea, Denmark and northern Gulf of Mexico, Florida. The lower capacity of egg cold storage is considered to link to the less seasonal variation in Florida, and the function of egg quiescent seemed to be suppressed. Second, artificial selection may take place when the eggs of Danish *A. tonsa* strain were stored repetitively over

generations in a long-term cultivation history (>25 years), the individuals innately have the superior adaptation in cold storage were selected and the overall capacity in egg cold storage was improved in this population (Drillet et al., 2007; Drillet et al., 2008). Third, the long-term cultivation under constant condition may cause genetic drift (Drillet et al., 2006a), and it may refer to the effect of domestication, which is an accumulative process over generations, and characterized by physiological or morphological alterations (Doyle, 1983).

Table 3.2 The comparison of the 48-h HSR of the eggs produced by long-term domesticated and newly-established culture strain of *A. bilobata*.

	48-h HSR over cold storage durations (%)					References
	Fresh	7 d	14 d	21 d	30 d	
Long-term domesticated strain	88.5 ± 1.9	89.4 ± 0.8	65.0 ± 3.1	53.7 ± 3.5	62.7 ± 5.4	Pan et al., 2014, Pan et al., 2015
Newly-established strain	66.5 ± 5.1	74.36 ± 5.13	85.8 ± 3.3	75.9 ± 5.2	62.1 ± 1.8	present study

The newly-established culture strain of *A. bilobata* was shortly domesticated (< 3 months), the high ratio of diapause production may reflect their original feature in natural habitat. Different from the out-door aquaculture ponds, copepods have less environmental stresses when they are cultured in the controlled and food-sufficient condition. In addition, the presence of predators in their natural habitat is reported to induce the occurrence of diapause in zooplankton (Hairston & De Stasio, 1988; Ślusarczyk, 1995; Pijanowska & Stolpe, 1996). Likewise, the elimination of predatory stress in the pure-species culture is likely to reduce the diapause production in *A. bilobata* over culture times.

In general, diapause egg is considered a mechanism to increase copepods' ecological fitness during the period of adverse environmental conditions. Temperature variation, one of the significant signals of seasonal change in nature, terminate the refectory phase meanwhile triggers the developmental resume in the copepod embryo. For instance, diapause eggs of two calanoid copepods *Labidocera aestiva* and *Eurytemora affinis* needed to experience the chilling before it hatch in the warming temperature (Marcus, 1980; Ban & Minoda, 1990). Conversely, the stimulation of warming on the diapause eggs of *Centropages hamatus* was required prior to the resume of embryonic development (Marcus & Lutz, 1998).

Although there is no previous study showing the relationship between temperature and diapause hatching rate of *A. bilobata*, the increased HSR found within the 14-d cold storage period implies that the chilling condition could trigger the hatching of diapause egg. We therefore conducted the additional experiment to examine the effects of different cold storage durations on the diapause HSR, and the HSR peaked in the 14-d treatment then followed by the gradual decrease with the extension of cold storage duration. This discovery suggested that most of diapause of *A. bilobata* has a short refractory phase in the cold condition. Interestingly, the increased number of unhatched diapause eggs occurred after the 14-d cold-storage, and these eggs stay undecomposed after the incubation of 72 h. Although we did not extend the incubation of the unhatched diapause, such eggs may be continually trapped in the refractory phase. We therefore raise a hypothesis here, if the refractory phase is a periodic cycle in copepod diapause egg. On the other hand, the diapause egg hatching could be possibly stimulated by other factors, such as drying (Bruno et al., 2001) and

photoperiodic change (De Stasio, 2004). Surely, the mechanism triggering the hatching of diapause eggs and the periodic hypothesis are held to be studied on the basis of copepod physiology and ecology.

In the batch of newly-spawned eggs, excluding the diapause and potentially unfertilized eggs, the rest of eggs hatched in under 72-h incubation were recognized as subitaneous eggs which could turn to be quiescent eggs at low temperature. Our estimation of the HSR in the quiescent eggs over cold storage durations was shown in Fig. 3.5. The estimated HSR of quiescent egg is around 53 % after 7-d cold-storage, which suggests around 80 % of subitaneous egg can switch into quiescent status. The estimated HSR of quiescent egg decreased with the increase of cold storage time, and it is related to the continuing metabolic activities in the eggs. The energy reserve was then slowly consumed during the cold-induced quiescence over time (Nielsen et al., 2006). The hatching success rate of quiescent eggs could gradually decrease with declining energy reservation to a point where they were even unable to hatch after long-term storage (Drillet et al. 2006a). Nevertheless, we also found the lower ability of transition between subitaneous and quiescent eggs in the newly-established culture strain used in the present study in comparison with the long-term acclimated culture strain in previous investigations (Pan et al, 2016). Similarly, we referred this is the phenotype of genetic diversity among populations, and the ability of quiescent induction may be improved with the extension of domestication and selective breeding. Indeed, production of copepod quiescent eggs is particular interesting for aquaculture due to their immediate induction and rapid hatching while well-incubated. Except the common cold storage technique used in the present work, other potential methods can be applied to improve the storage capacity of copepod quiescent eggs,

such administration of antibiotic and control of water quality (e.g. reducing sulphide concentration) in the egg storage container (Nielsen et al., 2006; Drillet et al., 2007). We have indicated the feasibility of cold storage technique for the egg of *A. bilobata*, and the studies on the improvement of egg storage capacity in *A. bilobata* are needed to be addressed to release their optimal potential for aquaculture uses.

Lipid has been long recognized to be an important way of copepod energy reservation, and copepod lipid are also significantly important for trophic ecology (Lee et al., 1971; Ratnayake & Ackman, 1979) and aquaculture nutrition (Drillet et al., 2011). Changes in lipid level have been reported in the diapause copepodites of many species of the genera *Calanus* and *Neocalanus* (reviewed in Lee et al., 2006). Those overwintering diapause copepods migrate to deep water after the active feeding season of spring or summer phytoplankton bloom, and they are characterized by the increased wax ester storage. The wax esters may allow diapausing copepodite to be neutrally buoyant and stay in the certain water layer called "diapause depth" (Visser & Jónasdóttir, 1999). Conversely, the dynamic of fatty acid composition in resting copepod eggs is scarcely addressed in field study, and it may be related to the problematic identification on species or dormant type of the eggs in sediment samples. Based on the different settling velocities and densities, resting and subitaneous copepod eggs are suggested being various in chemical composition (Marcus & Fuller, 1986). Few laboratorial studies have attained to assess the fatty acid level in the cold-stored eggs of *A. tonsa*, the changes of fatty acid level were detected even though it seemed to be minor over cold storage time (Støttrup et al., 1999; Drillet et al., 2006a; Sedlacek, 2008).

In this study, we analyzed the fatty acid composition of fresh and cold-stored eggs of tropical copepod *A. bilobata*. Different from the patterns reported in *A. tonsa*, we found the remarkable mobilization among the composition of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) in the egg of *A. bilobata* over different cold storage durations. The notable decline in MUFA and PUFA composition was detected in the 14-d cold storage treatment, which however had the peaked HSR. Currently, the functions of specific fatty acid on the HSR of copepod egg have not been clearly established. However, the dietary DHA level in the maternal generation can affect the HSR of egg (Evjemo et al., 2008), and it is suggested that copepod egg may require DHA reserve during the hatching although the direct analysis of fatty acid changes in the eggs was excluded from Evjemo's study. In addition, our analysis (Fig. 3.4) showed the negative relationship between DHA reserve (% total fatty acid) and the HSR of the cold-stored eggs, this implied the potential allocations or consumption of lipid reserve may occur in the copepod embryo during the pre-hatching phase. On the other hand, lipid-derived hormones have been reported to regulate the dormancy in copepodite copepod, a feedback system where the production of dormant hormones could be therefore expected to be linked to the fatty acid reserve in the eggs of copepod (Irigoien, 2004).

In conclusion, diapause and quiescence were identified in the resting eggs of *A. bilobata*. The production ratio of different resting types and cold storage capacity altered in different culture strains. The fatty acid composition can be the potential indicator of embryonic developmental stages for copepod resting eggs. Our findings contributed to the understanding of resting egg biology and ecology in tropical

copepod and also indicated a great potential of the cold-stored eggs of *A. bilobata* for aquaculture uses.

3.4.3 Photoperiod experiment

Photoperiod is one of the most significant seasonal signals in nature, and it could be a key impact affecting the reproduction of copepods. Several studies have focusing on the roles of photoperiod in regulating the diapause egg production (Macrus, 1980; Hairston & Olds, 1986; Hairston & Kearns, 1995; Avery, 2005; Glippa et al., 2013). The effects of photoperiod on copepod productivity have been, however, scarcely investigated (Peck & Holste, 2006; Camus & Zeng, 2008). In this study, we investigate the egg production and diapause production of the tropical calanoid copepod *A. bilobata* in different photoperiods.

The results showed that the egg production and diapause production of *A. bilobata* varied in different photoperiods. The significantly lower egg production revealed in the extreme photoperiod (24L: 0D, 0L: 24D), however, the significantly reduced production of diapause egg were found only in the full-dark condition (0L: 24D). The previous study reported that the egg production of the Baltic congeneric species *Acartia tonsa* was not affected by photoperiod (Peck & Holste, 2006). On the other hand, Camus and Zeng (2008) found the different result in their study focusing on another tropical congeneric species *Acartia sinjiensis*, where the egg production was remarkably higher in the long-day photoperiod. The effect of photoperiod on the egg production is likely to be species-specific, and it may be linked to the range of photoperiodic regimes can occur in the original habitat of the copepod species at various latitudes among seasons. In this work, the copepod *A. bilobata* was collected in

southern Taiwan, where they normally have the medium light and dark regimes. This may explain why the rather constant level of egg production was found in the medium photoperiodic treatments (16L: 8D, 12L: 12D and 8L: 16D).

We mentioned in the previous sections (3.4.1 & 3.4.2), copepod diapause eggs present ecological significance to regulate their population dynamic in the zooplankton community. As a function of seasonal variation, photoperiod is considered a cue of deteriorating condition for stimulating/modulating the formation of diapause eggs. The production of diapause egg increases when the maternal copepods experience increasing photoperiodic adversity. Interestingly, in our study addressed on *A. bilobata*, the significantly lowest diapause egg ratio was found under the full-dark treatment, but not in another extreme treatment of full-light. It has been reported that the photoperiod plays an important role in regulating the biochemical processes (e.g. endocrine and hormone regulations) in crustaceans (Nagaraju, 2011). However, there is currently no study establishing a relationship between photoperiod and diapausing or reproductive hormonal regulations in copepods, and it would be very interesting to be addressed in the future.

Chapter 4. Effects of cold selective breeding on the productivity, morphology and fatty acid content of the tropical cyclopoid copepod *Apocyclops royi*.

Pan, Y. J. *, Souissi, A., Sadovskaya I., Hwang, J. S., & Souissi, S (in prep.) A emerging approach of cold selection breeding on copepod *Apocyclops royi*: improving the productivity and fatty acid content.

4.1 Literature review

In marine larviculture, rotifer and brine shrimp are extensively used as prey for fish larvae rearing. These traditional live foods are highly available and easy to maintain in the hatchery, but their sub-optimal nutritional profiles have limited their support to the larvae production of many emerging aquaculture fish. On the other hand, copepods have been paid a great attention as potential live foods for marine larviculture (Støttrup & McEvoy, 2008; Støttrup, 2000; Conceição et al., 2010; Ajiboye et al., 2011; Drillet et al., 2011). Copepods have better nutritional suitability for marine fish larvae due to their superior content of essential polyunsaturated fatty acids (PUFAs) than *Artemia* and rotifer (Shields et al., 1999; Rajkumar & Kumaraguru vasagam, 2006; Piccinetti et al., 2014; Thuong & Hoang, 2015). Particularly, the rich content of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) of copepods are predominant in the form of phospholipids, which make the lipid of copepods more bioavailable (McKinnon et al., 2003; Bell et al., 2003). In addition, the small size (~100 μm) and swimming patterns of copepod nauplii offer a great palatability and higher predation attractiveness for small-mouth larval fish (Støttrup, 2000; Buskey, 2005; Mahjoub et al., 2011).

Although it has been well established that copepod supplementary diets can enhance the growth and survival of larval fish in many experimental studies (Payne & Rippingale, 2000; Evjemo et al., 2003; Olivotto et al., 2008a; Olivotto et al., 2008b; Barroso et al., 2013), the uses of copepods in commercial aquaculture facilities are still not common due to rather low productivity. The studies aimed to improve copepod production are therefore needed to release their optimal aquaculture

potentials. To select a suitable copepod species for feeding fish larvae is crucial and fundamental (Ajiboye et al., 2011), furthermore, the investigations of optimal culture conditions of the selected copepod species are required to maximize the productivity under artificial manipulation, such as photoperiod, salinity, density, temperature and diet (Peck & Holste, 2006; Camus & Zeng, 2008; Pan et al., 2014; Pan et al., 2016).

Selective breeding could be the effective approach for obtaining beneficial traits in aquaculture species (Gjedrem et al., 2012). Different methods have been performed on many species with economic interests, for instance, pacific white shrimp *Litopenaeus vannamei* (Argue et al., 2002), rainbow trout *Onchorhynchus mykiss* (Murata et al., 1996), red sea bream *Pagrus major* (Murata et al., 1996), channel catfish (Dunham, 2007) and Tilapia *Oreochromis niloticus* (Rezk et al., 2009). However, the selective breeding program has been scarcely addressed on copepods till the recent years (Lee et al., 2012; Alajmi et al., 2014; Souissi et al., 2014). A novel method of selective breeding based on the control of culture temperatures has been applied successfully to improve the physiological performances of the temperate calanoid copepod *Eurytemora affinis* (Souissi et al., 2014). To test the generality of the thermal selective breeding on other copepod species are important, here we therefore examined the responses of a very different copepod species *Apocyclops royi* which belongs to Cyclopoida and was collected from a tropical aquaculture pond. We acclimated two culture strains at critical low (18 °C, control strain) and given (28 °C, selective strain) temperature for 10 months (18 °C: around 15 generations; 28 °C: around 40 generations), then both strains were cultivated at 28 °C for 4 conductive generations. We attained to obtain the aquaculture-beneficial traits of *A. royi* by cold acclimation, and we conducted the mutigenerational observation to assess the

phenotypic plasticity and sustainability in the selective strain after facing temperature variation.

4.2 Material and Method

4.2.1 Copepod and microalgae stock culture

A pure strain of copepod *A. royi* was obtained from Tungkang Biotechnology Research Center of Taiwan. Stable cultures were established in the LOG-Marine Station of Wimereux, France for the experiments. Copepods were cultivated in 20-L polycarbonate carboys with diluted seawater at salinity 20 (made from a mixture of distilled water and 1-μm-filtered natural seawater). The cultures were placed in a photoperiodic room (12L:12D), and thermostatic heaters (EHEIM thermo control 50 W, EHEIM GmbH, Germany) were used to maintain the water temperature at 28 °C, which is referred to as the given temperature in their natural habitat.

The starter culture of microalgae *Isochrysis galbana* (Tahitian strain, T-ISO, Haptophyceae) was obtained from the Roscoff Culture Collection (Roscoff, France). Batch cultures of algae were performed in 2-L flasks containing autoclaved natural seawater supplied with Walne's medium (Walne, 1970) and maintained in the thermostatic incubator (MLR-351H; SANYO, Osaka, Japan) programmed at 18°C. The algae used for copepod feeding was in the exponential growth phase (3-4 days after inoculation). *I. galbana* was introduced every 2 days at the approximately cell concentration of 10^5 cells/ ml in the stock culture water of copepod, this feeding

concentration was determined to be the sufficient feeding level for *A. royi* due to the existence of live algal cells before every feeding.

4.2.2 Protocol of cold selection

The culture of *A. royi* was separated into two strains, control strain was kept cultivated at given temperature 28 °C as described previously (section 4.2.1). Conversely, the selective strain was transferred in the thermostatic incubator programmed at lower temperature 18°C, and this population was acclimated for 10 months (around 15 generations) until the experiment started.

4.2.3 Mutigenerational observation

Nauplii (N1-N2) were collected from both strains (at 18°C and 28°C), approximately 400 individuals were then transferred in each 2-L beaker (2 replicates per strain) to initiate the F1 generation (Fig. 4.1). The cultures were all maintained in the thermostatic incubator (MLR-351H; SANYO, Osaka, Japan) programmed at 28°C. Daily observations were carried out on the cultures, when the first ovigerous females occurred in the beakers, the cultures were kept for an additional 24 h to allow the females to release offspring. Subsequently, adult and nauplii were separated by sieving the culture water through 120 μm and 38 μm meshes. The nauplii were transferred in new culture beakers (2 L) for initiating culture of next generation. The adults were conserved at -80°C freezer for fatty acid analysis (protocol described in section 2.2.2), except of 25 ovigerous females that were gently sorted and cultured individually in 6-well cell culture plates (5 ml/ well) for an additional 24-h incubation. These females

and newly-hatched nauplii were collected and fixed in the buffered formalin solution (4%), and the nauplii production, body length of nauplii and adult female were then measured.

The morphological measurements were carried out under an inverted microscope (OLYMPUS IX71, Tokyo, Japan), using the software package Image J (version 1.41, National Institutes of Health, USA). The measurement of female length was performed from the top of cephalosome to the end of caudal rami, and nauplii length was realized from the longest part of nauplii. The biological traits (nauplii production, female and nauplii length, and fatty acid content) were documented during the F1 to F4 generation, except the fatty acid content was additionally examined in F0 population.

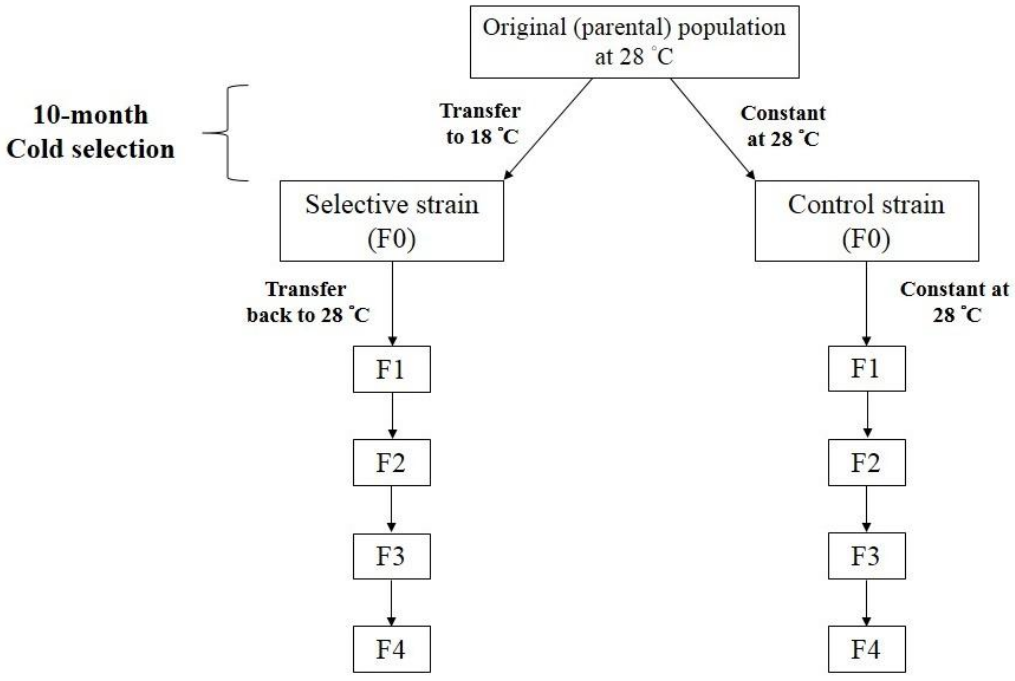

Fig. 4.1 Schematic overview of cold selection protocol used in the present study.

4.2.4 Data analysis

Calculation of current genetic gain

Current genetic gain (GC_C) is the value that expresses the level of proportional phenotypic change through artificial selection, and it is defined as the percentage change of biological traits between the progeny of control and selective strains (Van et al., 2014 ; Zheng et al., 2006), and it is calculated as

$$GC_C\ (\%) = \frac{(X_S - X_C)}{X_C} \times 100$$

where, X_S and X_C are the mean value of phenotypic traits of the selective strain and control strain, respectively.

Statistical analysis

An independent-samples t-test was used to compare the results of morphological and reproductive traits among control and selective strains within each generation with a defined level of significant difference ($P<0.05$). The data analyses of this study were run using SPSS program (SPSS, Chicago, IL, USA).

4.3 Result

4.3.1. Nauplii production

The nauplii production varied in selective and control strain among 4 consecutive generations (Fig. 4.2). The nauplii production of selective strain was all higher than the

control strain in every generation, and the significant difference was detected in F4. The nauplii production of control strain in different generations were as follows: F1: 17.8 ± 4.2 nauplii/ female, F2: 22.3 ± 6.7 nauplii/ female, F3: 20.6 ± 6.3 nauplii/ female and F4: 14.3 ± 3.6 nauplii/ female, and for selective strain were as follows: F1: 19.1 ± 3.2 nauplii/ female, F2: 22.4 ± 3.3 nauplii/ female, F3: 24.3 ± 6.9 nauplii/ female and F4: 18.5 ± 4.6 nauplii/ female.

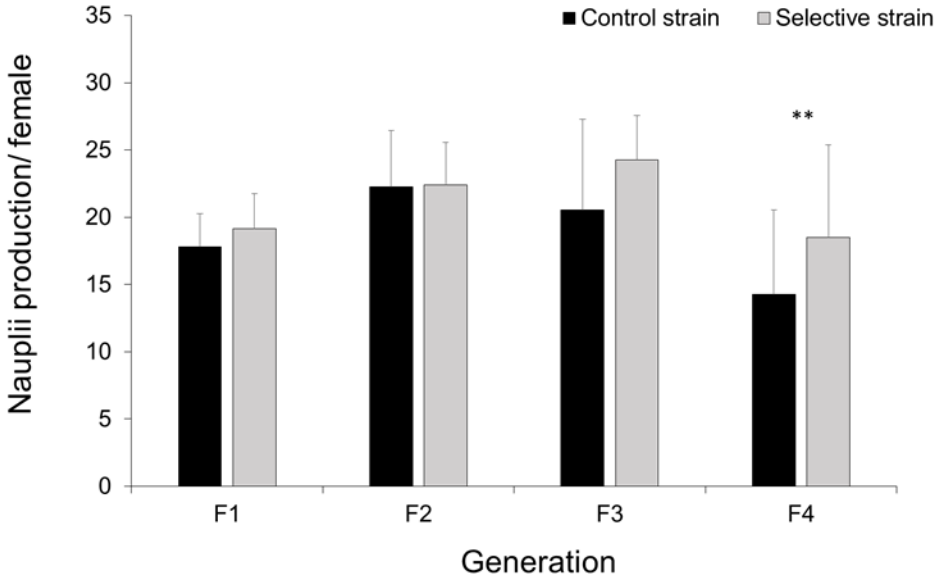

Fig. 4.2 Nauplii production of selective strain and control strain in different generations. Data are presented as mean ± SD, and the significant difference between control and selective strain within each generation is identified by independent-sample t-test, where ** indicates $p < 0.01$).

4.3.2. Female and nauplii size

Fig. 4.3 illustrates the length of adult female and N I nauplii of selective and control strains of *A. royi* in 4 consecutive generations. The females collected from the selective strain had higher body lengths than the control strain in all generations, and the

statistical significances were detected in F1 (control strain: 792.4 ± 15.3 μm; selective strain: 808.7 ± 19.0 μm), F3 (control strain: 792.4 ± 15.3 μm; selective strain: 862.2 ± 46.6 μm) and F4 (control strain: 752.8 ± 26.4 μm; selective strain: 845.0 ± 34.5 μm). For the nauplii size, the selective strain had significantly-lower lengths than the control strain in F2 (control strain: 113.3 ± 9.7 μm; selective strain: 107.4 ± 7.3 μm) and F4 (control strain: 114.1 ± 6.8 μm; selective strain: 108.5 ± 5.6 μm). Fig. 4.4 shows the relationship between female length and nauplii production, nauplii size, and the relationship between nauplii production and nauplii size of *A. royi*.

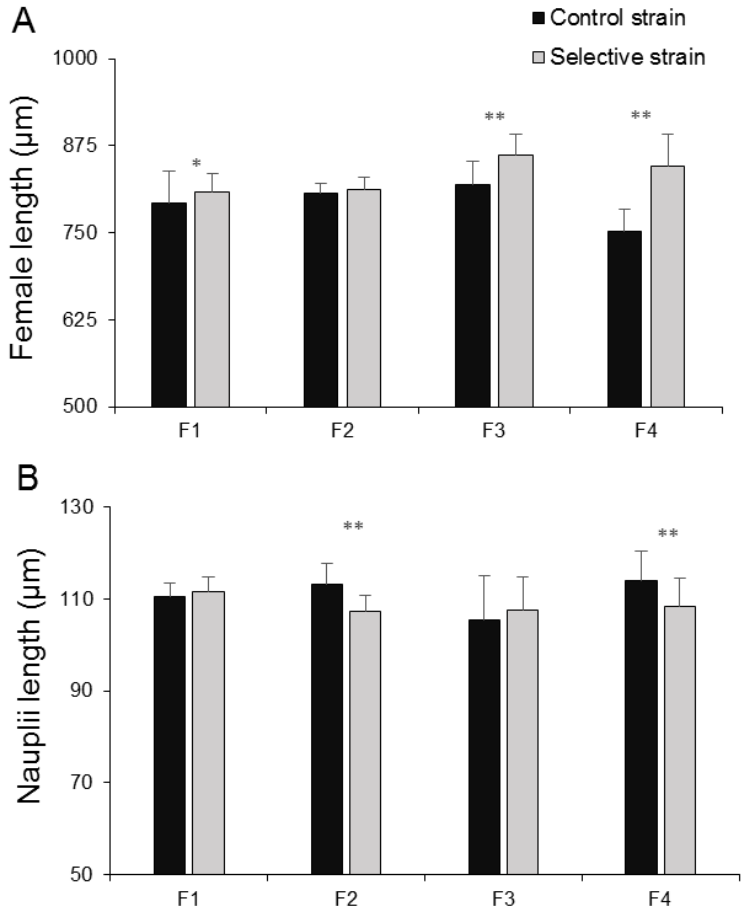

Fig. 4.3 Body length of (A) adult female (B) N I nauplii of the selective strain and control strain in different generations. Data are presented as mean ± SD, and the

significant difference between control and selective strain within each generation is identified by independent-sample t-test, where * indicates $p < 0.05$, ** indicates $p < 0.01$.

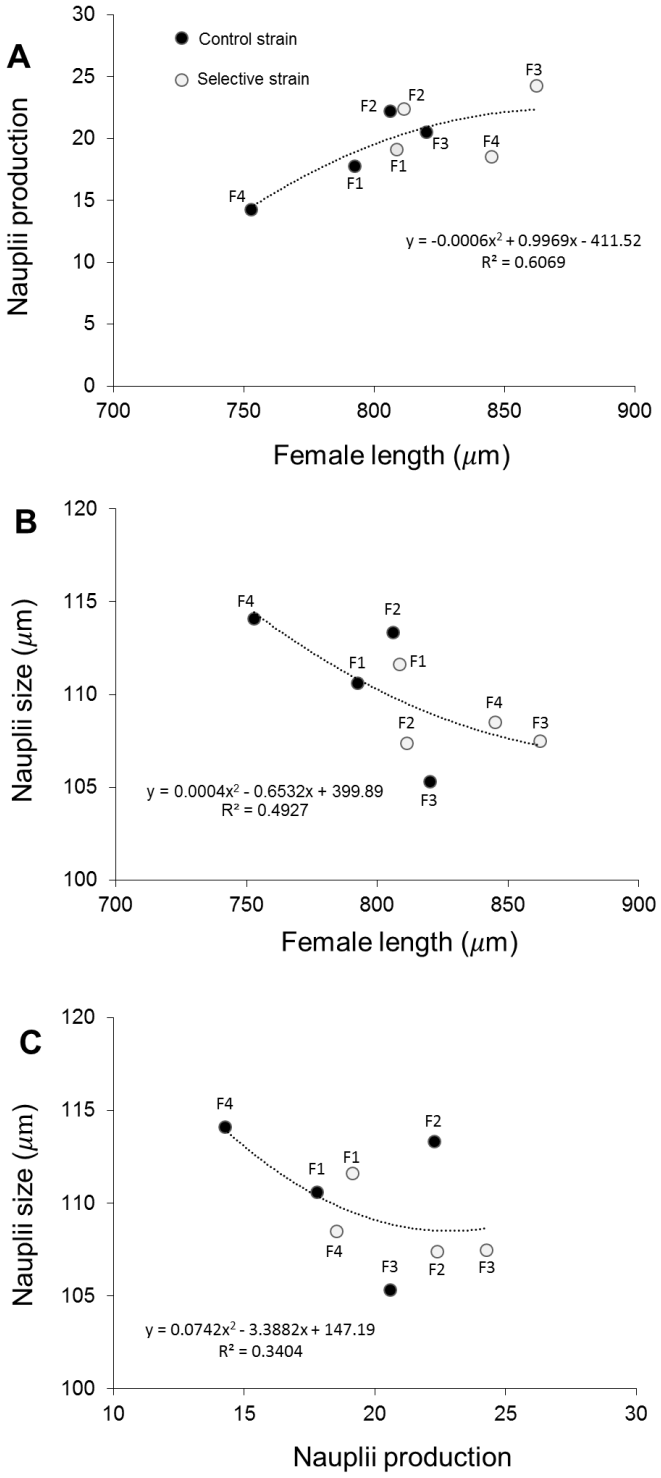

Fig. 4.4 Relationship between (A) female length and nauplii production (B) female length and nauplii size (C) nauplii production and nauplii size of *A. royi*.

4.3.3. Fatty acid content

The contents of fatty acid groups of control and selective strains in different generations are summarized in Fig. 4.5. Notably, the dramatic higher contents of n-3 PUFA, n-6 PUFA and total fatty acid were detected in the F1 of selective strain (40.1, 15.6 and 248.3 μg/ mg DW, respectively) than the control strain (16.7, 4.6 and 78.3 μg/ mg DW, respectively). The fatty acid content of selective strain gradually decreased in the following generations, and it was constantly identified to be higher than the control strain during F2 to F3. However, the lower contents of three fatty acid groups revealed in the selective strain than the control strain in F4.

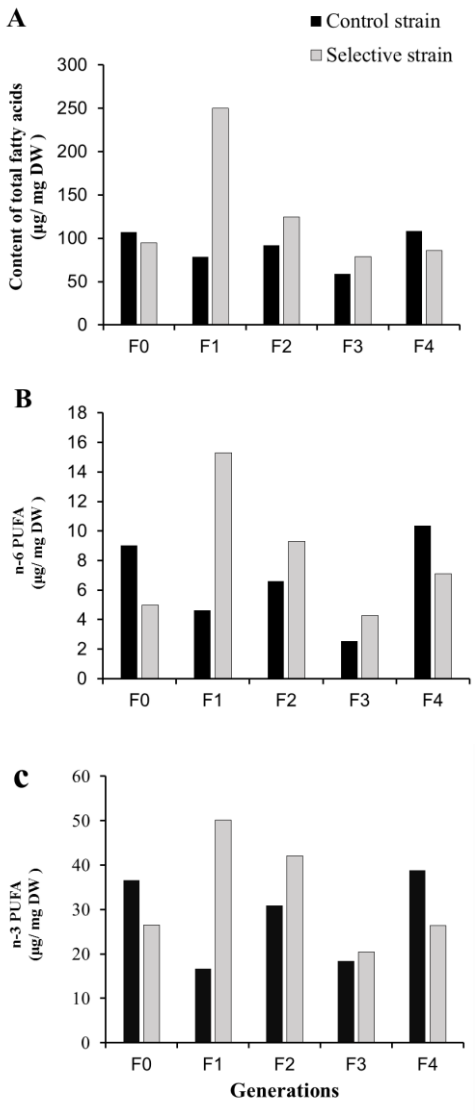

Fig. 4.5 Contents of (a) total fatty acid (b) n-6 PUFA (c) n-3 PUFA in the *A. royi* collected from selective strain and control strain in different generations.

4.3.4 Current genetic gain (GC_c)

To evaluate the efficiency of cold selection on *A. royi*, we caculate the current genetic gain (GC_C) to estimate the phenotypic variations of the selected strain on the basis of each generation (Table 4.1). It is clear that the cold selection can offer the advantages of increased female length and nauplii production among all generations, and the GC*c* were relatively significant in F3 and F4. The GC*c* of nauplii size is variable among generations, and the negative GC*c* were identified in F2 and F4. The huge GC*c* of total fatty acid, n-3 and n-6 fatty acid were obtained in F1, and the positive GC*c* remained in the selective strain except in F4.

Table 4.1 Current genetic gain (GC_C) of length of female and nauplii, nauplii production, content of n-6, n-3 polyunsaturated fatty acids (PUFA), and total fatty acid of cold-selective strain of *A. royi* in 4 generations.

Generation	Current genetic gain (%)					
	Female length	Nauplii length	Nauplii production	n6-PUFA	n3-PUFA	Total fatty acid
F1	2.1	0.9	7.6	85.5	211.4	218.3
F2	0.7	-5.3	0.6	10.0	34.5	33.8
F3	5.2	2.0	18.0	10.0	2.7	33.2
F4	12.2	-4.9	29.9	-9.4	-31.4	-20.1

4.4 Discussion

Selective breeding programs have been performed in the past few decades on many farmed fish, shellfish and brine shrimp for different goals, such as to improve growth rate (Zheng et al., 2006), enhance disease resistance (Argue et al., 2002), select

desirable body size (Shirdhankar & Thomas, 2003; Van et al., 2014) and increase body weight (Bondari, 1983; Rezk et al., 2009). However, the selective breeding on copepods has not been addressed until the first study focusing on cyclopoid copepod *Paracyclopina nana* in South Korea. Lee et al. (2012) evaluated the fecundity of six strains of *P. nana* collected from different habitats, and the strain with highest fecundity was further used for selective breeding. The high-fecund culture line was established by cultivating the isolated female *P. nana* with higher fecundity. Another selective breeding program was recently conducted with calanoid copepod *Parvocalanus crassirostris* in Australia, Alajmi et al. (2014) selected the females with higher reproductive capacity, and the enhanced egg production, size of egg, nauplii and female were achieved after 5 generations of selective breeding.

Similar to the majority of selective breeding programs in aquaculture, the two studies mentioned above were based on the selection of high performing individuals. However, the selection conducted at population scale based on environmental treatment seems to be rather simple to be performed at aquaculture facilities. Souissi et al. (2014) reported recently a novel approach of selective breeding based on thermal control in the population of temperate calanoid copepod *Eurytemora affinis*. The warm (20 ◦C) and cold (7 ◦C) strains were long-term acclimated, and both strains were then transferred to the increased temperature at 24 ◦C which was referred to be the applicable temperature for fish larviculture. After the cultivation of five generations, the cold-selective strain presented the overall larger body size, higher fecundity and presence of lipid droplet.

Temperature is one of the most important factors affecting physiological

performances of copepods, such as gene expression (Cailleaud et al., 2007; Schoville et al., 2012), metabolism (Gaudy et al., 2000; Ikeda et al., 2001), development, reproduction (Lee et al., 2003; Holste & Peck, 2006; Milione & Zeng, 2008; Devreker et al., 2009) and survival (Hall & Burns, 2002; Rhyne et al., 2009). Although the range of temperature tolerance is species-specific, adaptation may take place when copepods experience the exposure of temperature adversity (Bradley et al., 1988; Schoville et al., 2012; Dam, 2013; Souissi et al., 2016). Especially in populations with little gene flow, such as copepods living in geographical isolation (e.g. intertidal ponds and lakes) where local adaptation can occur (De Meester, 1996; Schoville et al., 2012). Studies have noted that the latitudinal-widespread copepods present various biological traits in different populations. Lonsdale and Levinton (1985) have observed the latitudinal cline for growth rate in populations of copepod *Scottolana canadensis* on the east coast of North America, where northern populations showed a higher growth rate at lower temperatures than southern populations. Similarly, remarkable alterations in gene expression were found in response to thermal stress in the latitudinal-separated population of copepod *Tigriopus californicus* in northern and southern California (Schoville et al., 2012).

Since temperature adaptation has been shown by the rapid response of thermal sensitivity in copepods, the temperature acclimations as the method of laboratorial selection for copepod strains seems to be feasible and promising. The egg-bearing cyclopoid copepod *A. royi* was used as our experimental model, this species can be found in tropical regions and it is used as the supplementary live food for larval groupers at some commercial hatcheries in Taiwan due to the high productivity and ease of maintenance (Su et al., 1997). Although *A. royi* has the wide range of

temperature tolerance (15 to 35 ◦C), the optimal culture temperature has been recognized in the high temperature range between 25 to 30 ◦C (Chang & Lei, 1993; Cheng et al., 1999; Hsu, 2000). However, to our knowledge, no study has reported the phenotypic variations of *A. royi* after long-term temperature acclimation. In our work, larger female lengths were constantly observed in the cold selective strain after it was transferred at 28 ◦C for 4 consecutive generations. Although the mechanism of thermal-size relationships during temperature acclimation of ectotherms have not been well concluded, the fact has been extensively observed that lower temperatures make ectotherms to develop slower, but mature at a larger body size (McLaren, 1965; Maly & Maly, 1998; Angilletta et al., 2004; Souissi et al., 2014). This relationship might be hypothesised to be a multivariate effect that consists of adaptive (genetic divergence) and nonadaptive plasticities (temperature-related alterations in biochemical processes) in ectothermal animals (Partridge & French, 1996; Van der Have & De Jong, 1996; Land et al., 1999; Angilletta et al., 2004). The increased body size of selected *A. royi* in our case can be expected as the result of this combinatorial hypothesis. However, the larger body length presented constantly after the population being transferred to the common temperature, the effect of genetic differentiation seemed to occur in the cold selective strain. Indeed, the temperature-related genetic divergence or modification of biochemical processes should be further investigated at molecular level in future studies.

Fecundity of copepod is a crucial paremeter often used to evaluate the aquaculture potential of copepod culture strain. Apart the constraint of food resource, copepod fecundity (in terms of nauplii production or clutch size) has been observed to increase with increasing body size in several studies (McLaren, 1965; Kiørboe & Sabatini,

1995; Poulin, 1995; Hirst & Bunker, 2003; Souissi et al., 2014). The positive relationship between female size and nauplii production was also observed in both strains (Fig. 4.4). Another concern for aquaculture application is the size of copepod nauplii due to the suitable prey size spectrum of larval fish. Aquaculturists have developed the great intention on the production of small-sized live preys to achieve the successful captive production of emerging farmed fish species with tiny larval mouth (People Le Ruyet et al., 1993; Fernández-Diaz et al., 1994; Chesney et al., 2005). The first stage nauplii (N I) of *A. royi* collected from both strains were in the size range of 105-115 μm, and it is smaller than the conventional live preys such as brine shrimp *Artemia* spp. (350-450 μm) and rotifer *Brachionus* spp. (150-360). The nauplii of *A. royi* offers an ideal size suitability for larviculture, nevertheless, the nauplii produced by the cold selected females are smaller than the control strain in all generations where F2 and F4 were identified to be significantly smaller. It has been generally observed that the copepod nauplii size is positively correlated to the egg size (Cooney & Gehrs, 1980). Furthermore, the egg size is less variable than adult body size when facing temperature variation (McLaren, 1965; Poulin, 1995), but it normally increases with increasing female size (Kiørboe & Sabatini, 1995). In the case of *A. royi*, the relationship between female size and egg size, however, are not observed in this study. It seems that the increasing body size of *A. royi* found in the cold selective strain might not achieve to certain physiological and morphological requirements to generate the larger naupliar copepods. On the contrary, we found the reduced nauplii size in the selective strain, and it is more likely to be linked to the increased fecundity (nauplii number) of female copepod (Cooney & Gehrs, 1980; Guisande et al., 1996) due to the reallocation in reproductive energy. In addition, according to our observation, female copepods from both strains required similar time

for maturity at 28 ◦C. The smaller N I developed to be larger adult female also suggested that the selective strain might have faster growth rate than the control strain.

To determine the lipid content of copepods is important for either trophic ecology and aquaculture application (Dalsgaard et al., 2003; Van der Meeren et al., 2008; El-Sabaawi et al., 2009; Rayner et al., 2015). In general, copepods live at high latitude (temperate or Arctic regions) have higher level of lipid reserve compared to the copepods distributed at low latitude (subtropical or tropical) (Farkas & Herodek, 1964). Laboratorial invetigation has suggested that the cold-acclimated copepods can increase the amounts of long chain fatty acid to maintain a standard membrane fluidity based on the hypothesis of homeoviscous acclimation (Nanton & Castell, 1999). Therefore, we assumed that the cold selective strain of *A. royi* may have the enhanced or recharacterized fatty acid value at 18 ◦C. However, we did not find the tendency of enhanced fatty acid content in *A. royi* after 10 months of cold acclimation at 18 ◦C (F0 population), conversely the slightly reduced lipid content was identified.

Although the detailed pathway of fatty acid synthesis in copepods have not been comprehensively summarized, the temperature-fatty acid relationship is generally considered linked to a species-specific temperature range for the active enzymatic system (Los & Murata, 1998). It is likely that, at 18 ◦C, the enzymatic activities for fatty acid synthesis of *A. royi* is suppressed, and this temperature may be below the minimal threshold for homeoviscous acclimation of the *A. royi*. The question of how *A. royi* maintain their biochemical functions on cell membrane without the reallocation of fatty acids in the extreme temperature condition still remains to be

answered. Interestingly, the dramatic increases of total fatty acid, n-6 and n-3 PUFA revealed in the F1 selective strain compared to F0 (2.5-fold, 3.2-fold and 1.6-fold, respectively, see Fig. 4.5). This elevated fatty acid content of the F1 population may be resulted in the metabolic compensation when the cold-acclimated copepods were transferred at their given temperature (Lonsdale & Levinton, 1985). In addition, the higher coefficient of varaition was estimated in the total fatty acid content among different genertations in the selective strain (CV: 56.3 %) than the control strain (CV: 23.5 %), which suggests that the copepod population may increase phsiological variations at generational scale to enhance the population fitness when they experienced the temperature change (18◦C to 28◦C). This physiological response to the thermal variation maintained shortly (two generations, F1 to F2) in the selective strain, which implies that the long-term cold acclimation on *A. roy*i is not necessary for improving the fatty acid content. However, this provides another prospective on the fatty acid self-enrichment by a short-term cold shock on *A. royi* before it is introduced to the warm-water fish larvae culture.

In conclusion, we confirmed that the cold selective breeding can improve the productivity and growth of *A. royi*, however, the huge acute increase in fatty acid content due to the metabolic compensation may not be sustainable. This work provides the understanding of reproductive biology and physiology in tropical copepod after facing temperature variation, and give an implication of potential method to improve the aquaculture application of *A. royi*.

Chapter 5. Feeding experiments of batfish *Platax orbicularis*, longsnout seahorse *Hippocampus reidi* and gilt-head bream *Sparus aurata*

5.1 Literature review

This chapter consists of three experiments which were contributed to two research projects based on the development of two pre-industrial pilots to produce copepods at large scale. The first project was initiated in 2012 in collaboration with the Aquarium Nausicaa in the framework of the project COPEFISH already presented in the beginning of this thesis. Recently in the framework of the second phase of COPEFISH a new experimental pilot was built in the spin-off space HALIOCAP in Boulogne sur Mer (agreement signed between the University of Lille 1 and the Communauté d'Agglomération du Boulonnais) under the supervision of Prof. Sami Souissi. This space was operated recently since early 2015 and allowed to focus only on the mass culture of microalgae and copepods using a home-made RAS (Recycling Aquaculture System). Consequently, the initial pilot space COPEFISH build in the tropical section of Nausicaa Aquarium, was managed differently to focus mainly on culture of fish larvae and the common living preys such as rotifers and Artemia. In the framework of the first phase of COPEFISH project two experiments on ornamental species batfish *Platax orbicularis* and longsnout seahorse *Hippocampus reidi* were performed. Recently, we used the Haliocap pilot to produce large number of quiescent eggs of *Acartia tonsa* in 3 x 300 liter tanks. Then, a series of experiments targeting larval rearing of seabream *Sparus aurata*, including those conducted in this thesis were realized in the COPEFISH facilities in Nausicaa in collaboration with the hatchery 'Ferme Marine du Douhet' (collaboration contract between this hatchery and Prof. Sami Souissi). This work was specifically aimed to investigate the feasibility to use quiescent eggs of *A. tonsa* as a live prey for gilt-head bream *Sparus aurata* which has extensive market demand for food consumption in Mediterranean countries.

Batfish *P. orbicularis* is a tropical reef-associated pelagic species that inhabits in the West-Pacific (Randall, 2005). The juveniles are known as the mimic morphology and behavior as fallen leaves or seaweeds (see Fig. 5.1). Therefore, the juveniles of *P. orbicularis* have the economic value of aquarium trade. In addition, adults of *P. orbicularis* are food consumption fish species in Taiwan, French Polynesia and other Tropical Pacific countries. This species is produced in small-scale aquaculture farms, however, the details of the first feeding patterns as well as the suitable diets have not been clearly reported in the previous literature. Thus, in the present study, the first feeding experiments were performed on the larvae of *P. orbicularis.*

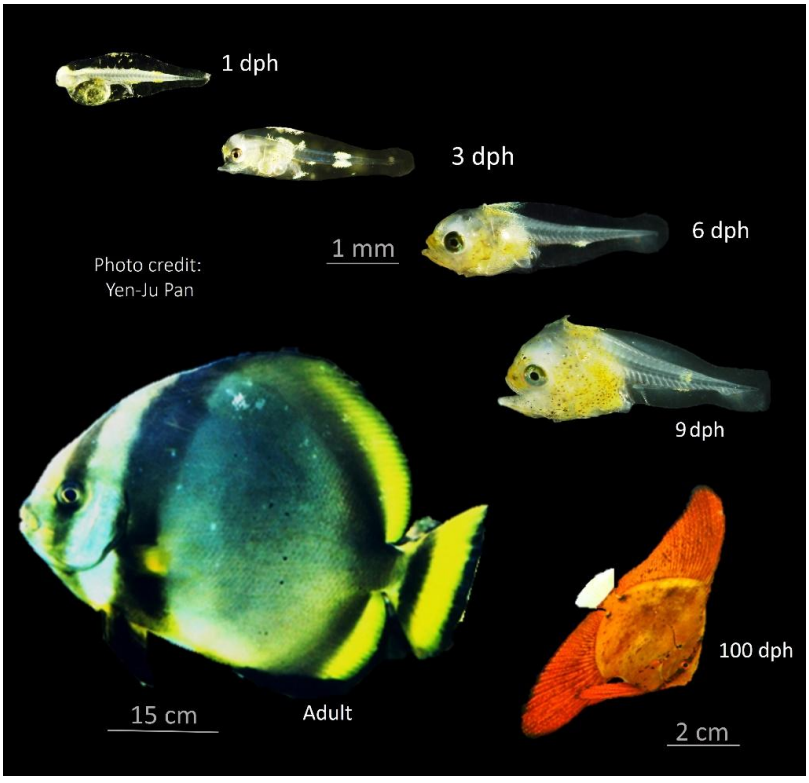

Fig. 5.1 Pictures of *P. orbicularis* in different developmental stages

Seahorses are strongly demanded in the aquarium trade worldwide, in addition, they are also utilized for traditional Chinese medicines. *H. reidi* (Fig. 5.2) is reported as a

threatened fish species due to their declining population in their natural habitats (Rosa et al., 2002), and it is recognized as a difficult species to be bred in aquaculture facilities (Giwojna, 2002). Olivotto et al. (2008a) reported the higher survival rate of *H. reidi* fed on harpacticoid copepod *Tisbe* spp. compared to the traditional diets composed with rotifer and Artemia. Olivotto's study suggested that copepods may be a potential diet for *H. reidi*, however, the different copepod species could have various effects on fish larvae feeding. Therefore, we used a very different diet, cyclopoid copepod *A. royi*, to feed the larval *H. reidi* in the present study.

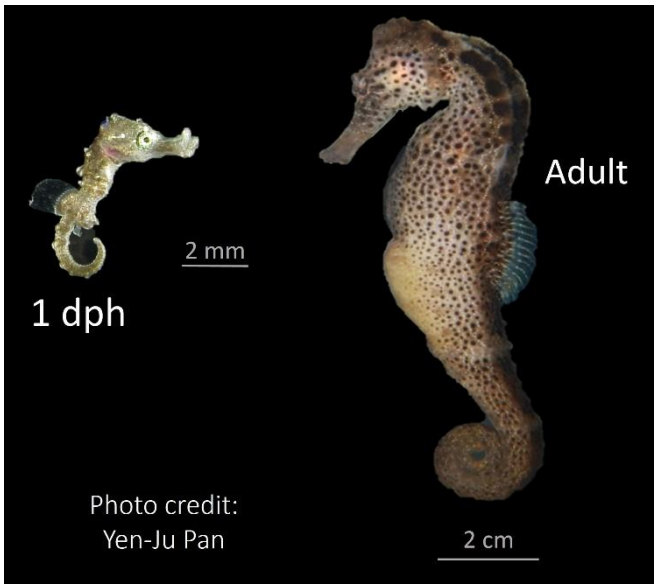

Fig. 5.2 Pictures of *H. reidi* in different developmental stages

Gilt-head sea bream (*Sparus aurata*, Fig. 5.3) is an economically-important fish species in the general Mediterranean aquaculture industry. This species has been well studied, therefore, we selected this fish species as our experimental animal to test the feeding effects of copepod nauplii (*A. tonsa*) derived from cold-stored eggs on the larval gilt-head sea bream.

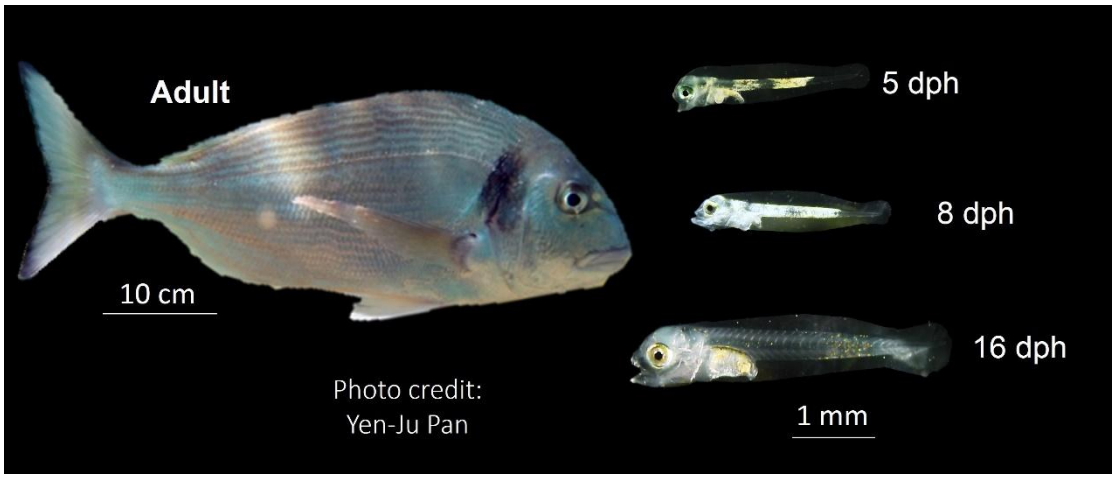

Fig. 5.3 Pictures of *S. aurata* in different developmental stages

5.2 Material and Method

5.2.1 Live prey preparation

Copepods

A. royi was cultivated in 70-L customized culture columns (Fig. 5.4.a) with diluted seawater (mixture of 1-μ-filtered natural seawater and tap water), and the cultures were fed with algae *I. galabana* at approximately concentration of 10^5 cells/ ml. The water temperature was maintained at 26-28 °C by a water heater and at the photoperiod of 12L: 12D cycle. The copepod in desired size were collected daily from the columns for larval feeding.

A. tonsa was cultivated in 300-L customized culture column (Fig. 5.4.b) with 1-μ-filtered natural seawater. The *A. tonsa*-based diets were prepared differently in the feeding experiments of *P. orbicularis* and *S. aurata* due to different feeding strategy and the availability of our lab facility. In the trial of *P. orbicularis* in 2014, the culture of

A. tonsa was maintained at the tropical section (COPEFISH working area), the cultures were maintained at 26-28°C and 12L: 12D cycle and fed with algae *Rhodomonas baltica* at approximately concentration of 8, 000 cells/ ml. The eggs were collected for larval feeding by siphoning the bottom of the column every day, and the eggs were subsequently introduced in the larvae tanks after being carefully cleaned.

In 2015, we used the *A. tonsa* nauplii derived from the cold-stored egg for feeding larval *S. aurata*. The cultures were maintained similarly as descripted above except the temperature was at 18 °C in our associated facility (Haliocap). The eggs were siphoned from the tank bottom and cold-stored at 4 °C followed the same protocol in section 3.2.2. The cold-stored eggs were cumulated during the period of 4 months until the start of the larval feeding experiment. To obtain the nauplii, the known number of eggs were incubated and enriched with algae *Isochrysis galbana* (Tahitian strain, T-ISO) for 24 h in the 40-L tanks. The nauplii were then collected for larval feeding.

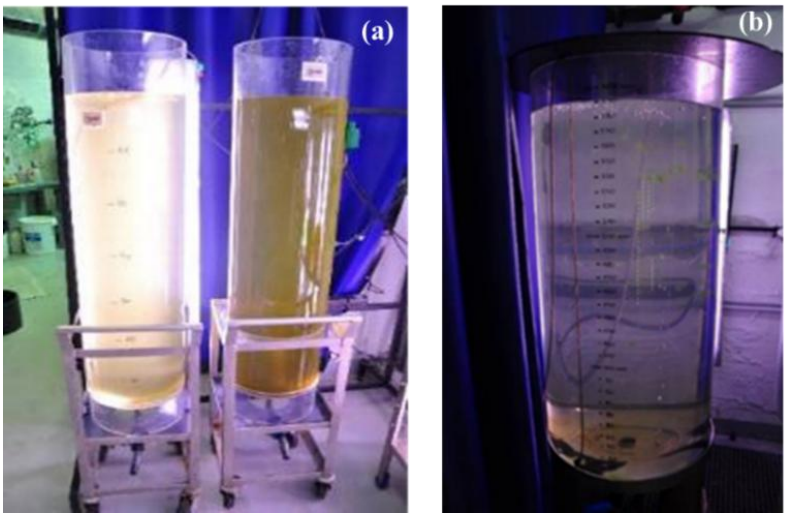

Fig. 5.4 The mass culture system of copepods (a) 70-L for *A. royi* (b) 300-L for *A. tonsa*

Artemia and rotifer

Rotifers were cultivated in 80-L and 150-L tanks, the cultures were fed with commercial product (ORI-Culture, Skretting) two times per day, and the culture water was completely changed every 2 day. Newly-hatched Artemia nauplii (N1) were obtained from the live prey section of aquarium Nausicaa. Both of these live prey were then enriched with commercial product (Selco S. presso, INVE AQUACULTURE) at slightly higher concentration than the manufacturer's instruction.

Fatty acid analysis

All the live preys used in this thesis were analyzed the fatty acid content/composition followed by the protocol mentioned in section 2.2.2, except egg of *A. tonsa*. Two samples of each live prey were collected on random date during the experimental period.

5.2.2 Feeding experiment of batfish *Platax orbicularis*

Natural spawning of the broodstock *P. orbicularis* was released in the exhibition tank "tropical lagoon" of Aquarium Nausicaa. The eggs of *P. orbicularis* were collected by the customized egg collector (333 μm plankton net), it was placed on the water surface (Fig. 5.5) where the artificial current make the water flow through the collector. The collector was installed every day at 15:00, and the eggs were harvested on every following day at 8:00. The eggs were placed in a container for 10 min, the floating eggs (the fertilized eggs) were collected and counted. Three to five hundred eggs were incubated in a 70-L black tank (approx. 3-7 larvae/ L). Three different treatments were designed (Table 5.1) as follows: 1. Control, 2. APO (*A. royi*-based diet) and 3. ACR *(A. tonsa*-egg-based diet). The final survival, weight and length of *P. orbicularis* were designed to be documented at the end of the feeding experiment (21 dph).

Table 5.1 Feeding protocol of *P. orbicularis* larvae

Feeding protocol of *P. orbiculari* experiment																				
Treatment	Live prey	Day post hatch (dph)																		
		3	4	5	6	7	8	9	10	#	12	13	14	15	16	17	18	19	20	21
1. Control	Rotifer	10 ind./ ml																		
	Artemia									5 ind/ml										
2. APO (*A. royi*-based)	Rotifer	10 ind./ml																		
	Artemia									5 ind/ml										
	A. royi	1 ind./ml (< 200 m)								1 ind./ml (>200 m)										
3. ACR (*A. tonsa*-egg-based di	Rotifer	10 ind./ml																		
	Artemia									5 ind/ml										
	Egg of *A. tonsa*	0.1 egg/ ml																		

Fig. 5.5 The egg collector in the exhibition tank "tropical lagoon" of Aquarium Nausicaa

5.2.3 Feeding experiment of longsnout seahorse *Hippocampus reidi*

Natural spawning of broodstock *H. reidi* was released in the 3x1 meter glass tank in Aquarium Nausicaa, the larvae were carefully collected and transferred to the larvae culture tanks. Around 100 larvae were cultured in the 30-L cage (approx. 4 larvae/ L) placed in the cubic tank (800 L). Two feeding treatments (1. Control and 2. APO; *A. royi*-based diet) were designed in this experiment (Table 5.2). The larvae were fed for 30 days, the survival and growth length and weight) were documented every 10 days.

Table 5.2 Feeding protocol of *H. reidi* larvae

Feeding protocol of *H. reidi* experiment																			
Treatment	Live prey	Day post hatch (dph)																	
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		to 30
1. Control	Artemia	5 ind/ml																	
2. APO (*A. royi*-based)	*A. royi*	1 ind./ml (< 200 m)								1 ind./ml (> 200 m)									
	Artemia								5 ind/ml										

5.2.4 Feeding experiment of gilthead bream *Sparus aurata*

The 2-dph *S. aurata* larvae were obtained from a French commercial hatchery (Ferme marine du Douhet). Around 3, 500 larvae were placed in each the 70-L tank (approx. 50 larvae/ L) for 12-h acclimation, the live prey was introduced since 3 dph. Two feeding treatments (1. Control and 2.ACR; *A. tonsa*-nauplii-based) were designed in this experiment (Table 5.3). The lengths of larvae were designed to be documented on 2, 8, 19 dph, and the final survival rate were estimated at the end of the experiment.

Table 5.3 Feeding protocol of *S. aurata* larvae

Feeding protocol of *S. aurata*																	
Treatment	Live prey	Day post hatch (dph)															
		3	4	5	6	7	8	9	10	#	12	13	14	15	16	17	18	19
1. Control	Rotifer	8 ind./ml			6 ind./ml						4 ind./ml							
	Artemia										0.5 ind/ml							
2. ACR (*A. tonsa*-nauplii-based diet)	Nauplii of *A. tonsa*	8 ind./ml																
	Rotifer					6 ind./ml					4 ind./ml							
	Artemia										0.5 ind/ml							

5.3 Results

5.3.1 Fatty acid profiles of live preys

Table 5.5 shows the fatty acid profiles (composition and content) of the enriched Artemia, enriched rotifer, naupliar *A. tonsa* derived from the cold-stored eggs and *A. royi* (*A. royi* data refer to section 2.2.3). The highest total fatty acid content was found in the enriched Artemia (136.87μg fatty acid /mg DW biomass) followed by *A. royi* (87.83 μg fatty acid /mg DW biomass) and enriched rotifer (71.69 μg fatty acid /mg DW biomass), and the lowest fatty acid content was found the naupliar *A. tonsa* derived from the cold-stored eggs (27.24 μg fatty acid /mg DW biomass). The composition of fatty acid also

varied among live preys. The highest composition of total saturated fatty acid was found in *A. royi*, the highest composition of monounsaturated fatty acid was found in Artemia and the highest composition of n-6 PUFA was found in rotifer. Notably, the naupliar *A. tonsa* had the highest proportion DHA and EPA as well as the total n-3 PUFA.

5.3.2 Feeding experiment of batfish *P. orbicularis*

Different number of replicates were examined among three diets due to the uncontrolled spawning event of the broodstock. We conducted 5 replicates in control and APO treatment, and 2 replicates in ACR treatment. The unexpectedly sudden mortality occurred in all the replicates of three treatments, which did not allow us to collect a sufficient number of larvae for morphological measurement even we stopped the experiment much earlier (7-14 dph) than we designed. Except the only two complete observations were accomplished each in control and ACR treatment (Table 5.4). The 21-d survival rate in control and copepod egg-*A. tonsa* were 2.49 % and 1.94 %, respectively. Although the survival was slightly lower, the higher body length, weight and metamorphosis rate were found in ACR treatment.

Table 5.4 The survival rate, metamorphosis rate, body weight and length of *P. orbicularis* larvae fed on different diets.

	Survival (%)	Meta. rate (%)	Body weight (g)	Body length (cm)
Control	2.49%	83.33%	0.08	1.36
ACR	1.94%	100.00%	0.11	1.61

Table 5.5 Fatty acid profiles of enriched rotifer, enriched Artemia, *A. royi* and naupliar *A. tonsa* (hatched from cold-stored eggs). nd = not detected, SFA = Saturated fatty acids, MUFA = Monounsaturated fatty acids, PUFA = Polyunsaturated fatty acids, ARA= Arachidonic acid, ALA= alpha-Linolenic acid, EPA= Eicosapentaenoic acid, DHA= Docosahexaenoic acid.

	Rotifer	Artemia	nauplii *A. tonsa*	*A. royi*
µg fatty acid / mg DW	71.69	136.87	27.24	87.83
fatty acid composition (% total fatty acid)				
SFA				
C14:0	0.88	0.62	1.91	3.64
C15:0	0.45	0.13	0.35	0.38
C16:0	16.08	12.91	16.88	19.39
C18:0	7.16	5.34	10.10	11.01
C20:0	nd	nd	nd	nd
C21:0	nd	nd	nd	1.42
MUFA				
C16:1	2.10	2.30	1.20	3.54
C18:1	14.20	32.96	5.03	16.30
C20:1	2.64	nd	nd	nd
n-6 PUFA				
C16:2 n-6	0.23	0.17	nd	nd
C18:3 n-6	nd	nd	nd	0.11
C18:2 n-6	12.01	8.28	1.99	6.86
C20:4 n-6 (ARA)	1.42	1.00	0.22	1.34
C20:3 n-6	0.52	0.00	0.00	nd
C22:5 n-6	1.42	1.71	1.32	2.28
n-3 PUFA				
C18:4 n-3	0.00	0.00	8.35	6.91
C18:3 n-3	1.64	8.77	7.25	3.68
C20:5 n-3 (EPA)	5.29	6.86	13.76	3.12
C20:4 n-3	1.01	0.00	0.00	1.02
C21:5 n-3	1.00	0.00	0.00	nd
C22:6 n-3 (DHA)	25.42	17.96	30.82	18.99
C22:5 n-3	5.99	2.86	0.82	nd
ΣSFA	25.11	19.01	29.24	35.83
Σ MUFA	18.94	35.26	6.23	19.85
Σ n-6 PUFA	15.60	11.17	3.52	10.59
Σ n-3 PUFA	40.35	36.44	61.00	33.73
Σ PUFA	55.95	47.60	64.52	44.32
DHA/EPA	4.81	2.62	2.24	6.09

5.3.2 Feeding experiment of longsnout seahorse *H. reidi*

The survival rates of *H. reidi* larvae fed on different diets were shown in Fig. 5.6. We conducted 5 replicates for control treatment and 4 replicates for *Apocyclops*-co-feeding treatment. The averaged 30-d survival rate of control treatment (25.4 %) was higher than the *Apocyclops*-co-feeding treatment (15.7 %). For morphological traits (Fig. 5.7), the higher body height and wet weight of 30d-old larvae were found in the control treatment (height: 25.2 mm, wet weight: 46.4 mg) than in the co-feeding (height: 22.0 mm, wet weight: 25.9 mm).

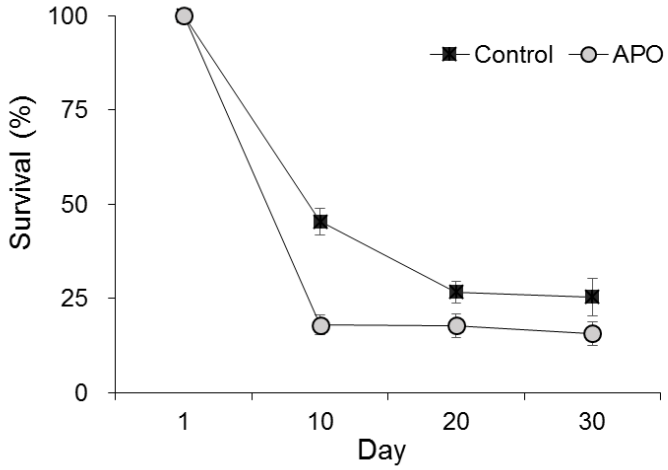

Fig. 5.6 Survival rate of *H. reidi* fed on different diets.

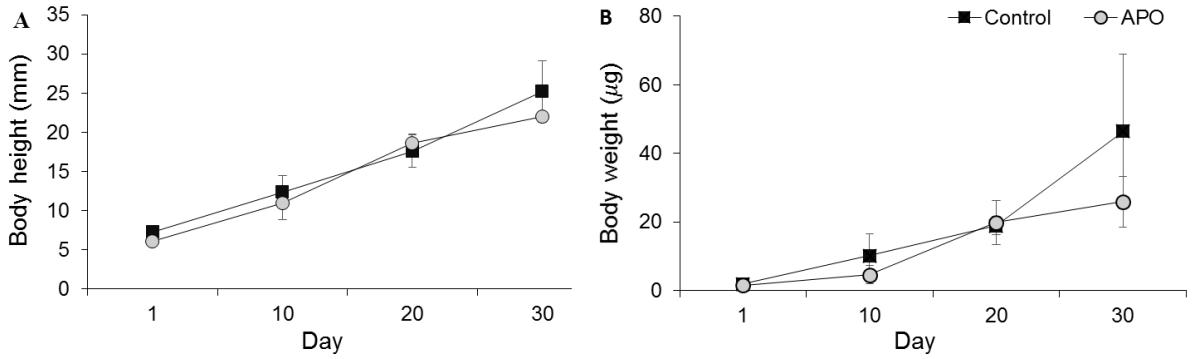

Fig. 5.7 (A) Body height and (B) weight of *H. reidi* fed on different diets.

5.3.3 Feeding experiment of gilt-head bream *S. aurata*

The final survival rate of larval gilt-head bream was illustrated in Fig. 5.8, where showing the markedly higher survival in copepod treatment (8.3 %) than in the control (1.4 %). Fig. 5.9 shows the size of larvae fed on different diets on 2, 8 and 19 dph. Before the first feeding, the larvae measure 3.29 ± 0.18 mm, then increased to 3.41 ± 0.24 mm in the control and 3.55 ± 0.25 mm in the ACR treatment. At the end of observation, the 19-dph larvae are markedly larger in ACR treatment (5.54 ± 0.54 mm) than in the control (4.66 ± 0.59 mm).

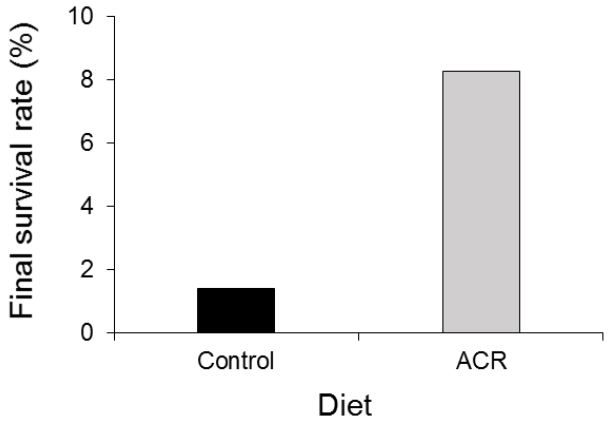

Fig. 5.8 Effects of different diets on the survival rate of 19-dph larval *S. aurata*.

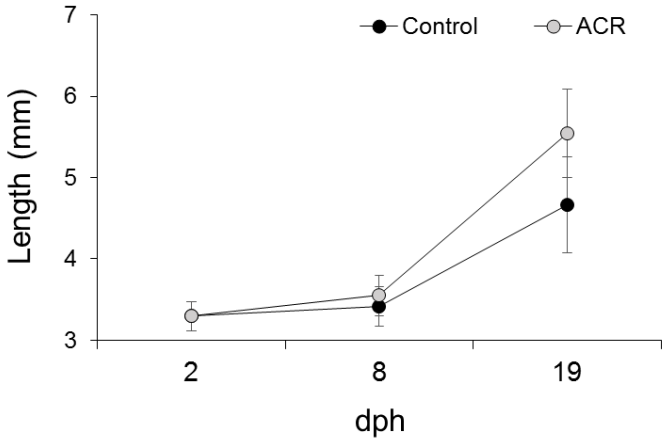

Fig. 5.9 Effects of different diets on the size of *S. aurata* larvae fed on different diets on 0, 8 and 19 dph.

5.4 Discussion

This chapter was aimed to investigate the effects of different copepod-based diets on the first feeding of marine fish larvae. In 2014, we started the experiments on the tropical pelagic fish *P. orbicularis* which was also the continuity of my work during pre-doctoral study (summer of 2012). Although *P. orbicularis* has been successfully captive bred at small scale in our preliminary trial in 2012 and also at extensive scale in other aquaculture facilities (French Polynesia and Taiwan) (Chiu et al., 2014; Gasset & Remoissenet, 2011), the details of optimal culture protocol (water temperature, water exchange ratio, light intensity and photoperiod…etc.) of this species has not been well established in the literature. And the information of culture techniques of such species with great commercial potential seem to be very difficult to be obtained. Therefore, we also conducted several pre-tests to develop the suitable culture protocol before the feeding experiment had been launched.

Apart from conventional live prey (rotifer→Artemia) as control, we initiated the feeding experiment with the co-feeding diet based on the tropical cyclopoid copepod *A. royi*. This copepod species can be found in sub/tropical Indo-pacific area, where is also the natural habitat and the majority of aquaculture farms for *P. orbicularis* located. Moreover, *A. royi* has been used as food supplement for larval groupers at commercial hatcheries in Taiwan (Su et al., 1997). The result revealed, however, that the larvae of *P. orbicularis* presented extremely low survival rate when fed on either control or *A. royi*-based diet in our study. Along with the low initial larvae number, we encountered difficulty having enough larvae for the desired assessments of any sorts. In addition, the broodstock was not controlled in this study, the variable egg production (quantity and

spawning period) created technical difficulties to optimize our experimental schedule. Interestingly, we obtained one complete set of trial (21d) from our last available larvae cohort fed on fresh-spawning egg of *A. tonsa*, such copepod has been long recommended as the suitable live prey for larviculture in Europe (Støttrup et al., 1986). The increasing body length, weight and metamorphosis rate indicated that the diet based on *A. tonsa* egg can enhance the culture performances of *P. orbicularis*, and this preliminary test encouraged us to use the egg of *A. tonsa* in our later fish larvae trial.

At the end of *P. orbicularis* spawning season, we occasionally obtained the spawning of the seahorse *H. reidi* in the Aquarium Nausicaa, we therefore conducted the additional experiments to test the effects of *A. royi*-based diet on the seahorse larvae. The larvae of *H. reidi* showed much higher resistance than *P. orbicularis*, and it allowed us to complete the 30-d first feeding experiment with replicates. In the previous study, Olivotto et al. (2008a) used a *Tisbe*-based diet (harpacticoid copepod) and control diet (rotifer→Artemia) for feeding the larvae of *H. reidi* under two different photoperiods (natural 14L: 10D, constant light 24L: 0D). Although they found the higher survival rate and growth in the constant light condition, here we would only discuss the effect of diets on the larvae. Under the condition of natural photoperiod (12L: 12D), the larvae fed on control diet (rotifer→Artemia) showed 10 ± 1%, and on *Tisbe*-based diet showed 14 ± 1% of 21-d survival rate. The *Tisbe*-based diet can result in a higher survival, Olivotto et al. however suggested that the benthic copepods such as *Tisbe* spp. may not be suitable for feeding the seahorse.

According to our preliminary test, the 1-d larval *H. reidi* have the ability to capture Artemia nauplii, and the rotifer seemed to be barely attractive for them to feed on. We

used solely the Artemia in the control diet and we eventually obtained the higher final survival rate (25.4 %) after the longer experimental period (30 d) than the research of Olivotto (10 ± 1%, 20d). However, the lower survival rate (15.69 %) was found when the larvae fed on *A. royi*-based diet than our control. A successful prey capture by fish larvae could be linked to several reasons, such as prey size, visual detectability and swimming velocity. The swimming pattern of *A. royi* was reported to be very different from calanoid copepod (e. g. Genus *Acarti*a and *Pseudodiaptomus*). Wu et al., 2011 have reported *A. royi* has the rapid and jerky swimming pattern. In addition, *A. royi* showed longer detection distances to food because of their longer setae and higher mechanosensory abilities, and this species is able to perceive motile food organisms at larger distances. Based on Wu's research, it can be expected that *A. royi* has the higher ability to detect and avoid the predator. Therefore it do not allow the larval *P. orbicularis* and *H. reidi* to capture them efficiently, which possibly makes the larvae spend excessive energy on predation and result in the reduced larvae survival.

In 2015, we selected the well-studied and extensively-produced aquaculture species gilt-head bream *S. aurata* as our experimental model. From the inspiration obtained in *P. orbicularis* trial, we developed the new feeding strategy where the nauplii derived from the cold-stored eggs of *A. tonsa* were used as prey. The cold-stored *A. tonsa* egg has been suggested to be the potential live prey for larviculture (Drillet et al., 2006b). However no study has well documented the actual effect of such diet on larvae feeding. In order to increase the feeding density of nauplii, the eggs were produced and cumulatively conserved in cold condition for 4 months prior to the experiment. The known-number egg batch were incubated and enriched with algae *Isochrysis galbana* (Tahitian strain, T-ISO) for 24h, and the averaged HSR was estimated at 55.8%. We

conducted two replicates per treatment (control and ACR), and the higher averaged final survival rate and body length of the gilt-head bream *S. aurata* larvae showed in the copepod-nauplii-based diet than the control.

Although the lower actual content of fatty acid was detected in the nauplii derived from cold-stored eggs of *A. tonsa* than the other diets (Table 5.5), the highest composition (% total fatty acid) of DHA, EPA and total PUFA may support the survival and growth of larval sea bream. It has been stated that the copepod fatty acid is dominant in the form of phospholipid, and it allows the fish larvae to digest and absorb it more efficiently (Evjemo & Olsen, 1997). On the other hand, the micronutrients such as vitamins and minerals are also reported to be important for fish larvae nutrition (Dantagnan et al., 2016 ; Merchie et al., 1997), and copepods have been reported to have rich content of such elements (Rønnestad et al., 1998 ; Watanabe et al., 1983). Obviously, the content of selected micronutrients would be interesting to be confirmed in the future studies.

Here we summarize our findings on the feeding suitability of two copepod species on different fish. The cyclopoid copepod *A. royi* provides fair content and composition of fatty acid (Table 5.5, and see section 2.4.1), but it is likely to be the sub-optimal prey for larval *P. orbicularis* and *H. reidi* due to their swimming velocity and pattern. The naupliar *A. tonsa* derived from the cold-stored eggs offer the favorable fatty acid composition where contains high proportion of DHA, EPA and total PUFA, and it can be a supportive diet for enhancing the survival and growth of *S. aurata* larvae. Based on our results, we recommend that *A. tonsa* nauplii derived from the cold-stored eggs could be a beneficial supplementary diet for pelagic fish larvae (*P. orbicularis* and *S. aurata*). The copepod *A. royi* contains also great nutritional values, however, the use of

this species for larvae feeding should be carefully evaluated/ decided by considering the swimming ability of fish larvae.

Chapter 6. General conclusion and perspectives

6.1 Copepod cultures

I have been focusing on the research of copepod biology and aquaculture since 2010 when I started my master degree. In the past 6 years, I investigated the appropriate technique for cultivation, egg cold storage and selective breeding for two key copepod species *A. bilobata* and *A. royi*, and here I conclude the major findings of my work and give perspectives for the future studies.

6.1.1 Food and nutrition

From the results obtained in this PhD thesis (chapter 2) and my master thesis (Pan et al., 2014), I found that the cyclopoid copepod *A. royi* is more accepting to various microalgae cells with different cell size and structure in the late developmental stages (copepodite/adult) than the calanoid copepod *A. bilobata*, but the larger-sized microalgae (e.g. *T. cui*, around 15 μm) still create limitation for the feeding of naupliar *A. royi*. In chapter 2, I used the compositional relationship of fatty acids between prey and predator to explain the fatty acid requirement of copepods. The cyclopoid copepod *A. royi* has the ability to endogenously synthesize the long-chain PUFAs (C20 and C22) from shorter chain PUFA (C16 and C18), and the calanoid copepod *A. bilobata* is likely not to have this ability. Among three selected microalgae species, *I. galbana* offers the overall greatest nutritional advantages (high and balanced content in DHA and EPA) and size suitability (3-6 μm) for both copepods.

To summarize, the small-sized microalgae species (3-6 μm) with rich content of long-chain essential fatty acids are preferable for both copepod species, this finding

provides the principle of food selection for optimizing the feeding of *A. royi* and *A. bilobata* for multidisciplinary purposes.

For the future, as it is scarcely addressed, the detailed metabolic mechanism of fatty acid in copepods should be studied at molecular level. The prospective study will be aimed to develop the reliable molecular markers in fatty acid synthesis pathway, and to determine how the dietary fatty acid modulate the fatty acid synthesis of copepods. This potential work would further contribute to the understanding of trophic ecology and the development of potential method of enrichment for aquaculture uses.

6.1.2 Salinity and photoperiod effects

The cyclopoid copepod *A. royi* has been observed to have the wider range of salinity tolerance than *A. bilobata*, so it was selected as live prey to feed marine fish larvae in this thesis (chapter 5). In order to verify the most suitable salinity for culturing *A. royi*, and what would happen when the copepods are introduced in the fish larvae tank among potential salinities for aquaculture, the reproduction of *A. royi* at either individual and population scales were investigate. Overall, I confirmed that 20 is the optimal salinity for attaining the highest production of *A. royi*, and salinity had varying influences on *A. royi* across its different developmental stages. Female *A. royi* reduces the egg production at low salinity (0-10), on the other hand, the result suggested the high mortality of nauplii at high salinity (30-35) without altering the egg production. For aquaculture application, it is feasible to mass culture *A. royi* at medium salinity (15-20) and collect the nauplii for fish larviculture in different salinities. However, due to the low survival of naupliar *A. royi* at high salinity, the more frequent feeding

administration for marine fish species would be needed to allow higher live prey availability and quality. The salinity effects on mobility of chemical composition (in terms of nutritional value) of *A. royi* would be interesting to be focused in the coming studies, in particular the content of amino acid is closely related to the intercellular osmoregulation. In addition, a long-term salinity acclimation would be carried out to elucidate the salinity adaptation, and to select the potential strain at desired salinity for aquaculture purpose.

The effects of photoperiod on the overall egg production and the ratio of different egg types on the free spawning calanoid copepod *A. bilobata* were examined. The constant level of egg productions were found in the medium photoperiodic treatments (16L: 8D, 12L: 12D and 8L: 16D), and the significant lower egg production showed in full light and dark conditions. This may be related to the natural photoperiod range occurs in the original habitat of *A. bilobata.* Interestingly, the diapause egg production ratio remain stable except in the full-dark treatment. The light regime has been noted to be an important regulator/stimuli for the biochemical processes (e.g. endocrine and hormone regulations) in crustaceans (Nagaraju, 2011). Based on our finding, it can be expected that photoperiod may be involved in the diapausing or reproductive hormonal regulations in copepods, and it would be very interesting be released in the future studies at molecular level.

6.1.3 Egg cold storage

This study reveals for the first time the viability of artificially cold-induced quiescent eggs for the tropical calanoid copepod *A. bilobata.* Based on the egg hatching

characteristics, we classified two resting types (diapause and quiescence) in the egg of *A. bilobata*. Notably, the production ratio of various egg types and the cold storage capacity altered in different culture strains, and it seems depending on the level of culture domestication. We also confirmed that the fatty acid composition altered during the cold-storage period, and it could be regulated by the potential feedback system where the dormant hormones is likely to be linked to the fatty acid level in copepods (Irigoien, 2004).

The "strain effect" on the production ratio of different egg types and cold storage capacity would be kept following in the future as long as the newly-established strain of *A. bilobata* is available. And it would be needed to study on how to improve the capacity of egg cold storage in many potential ways. For instance, to reduce the stresses of deteriorating water quality (e.g. sulphide density) or to reduce pathogen by antibiotic or probiotic treatments in storage tubes. Finally, the relationship between fatty acid level and regulation of dormant hormones in copepod eggs would be very arousing and original to study, which may contribute to the eco-physiological understanding of copepod egg dormancy, and also could provide a potential cue on using fatty acid enrichment to enhance the cold storage capacity.

6.1.4 Selective breeding

The method of cold selective breeding was firstly used to improve the physiological performances of the temperate calanoid copepod *E. affinis* (Souissi et al., 2014). In this study, I performed the selective breeding on the tropical cyclopoid copepod *A. royi* by different temperature switch (28°C →18°C→28°C). The constant larger female size

and higher nauplii production were obtained in the selective strain. The compensative enhance of fatty acid content showed in the first generation when the selective strain was transferred back at given temperature (28°C), but it was not sustainable in the later generations. Although this work was initiated to improve the aquaculture potential, we found the temperature adaptations of copepods could vary in different copepod species. The continuity of this work can be focused on the changes of biochemical processes in copepod species among orders and different climate conditions, this can contribute to the understanding of copepod eco-physiology and how they response to the global warming in the future. In addition, to understand the different patterns of temperature adaptations in various copepods may offer the ideas on functional evolution of copepods.

6.2 Feeding experiments of fish larvae

The fish larvae were sensitive and fragile to be cultivated in our pilot larvae rearing facility. Our recent investigations showed that the potential pathogens (*Vibrio.* spp.) and heavy metal contamination were identified in the seawater at our culture facility (Zidour et al., unpublished data). And it could be the potential risk leading the general low fish larvae survival observed in our experiments. The conditions of culture system (water and air quality, light spectrum and pathogen control…etc.) would be monitored and improved in future trials.

Based on the positive results obtained in the feeding experiments of *P. orbicularis* and *S. aurata*, we recommend that the naupliar *A. tonsa* derived from the cold-stored eggs could be the beneficial supplementary diet for pelagic fish larvae. The higher growth

and metamorphosis rates were found in the larvae of *P. orbicularis* and *S. aurata* fed on the *A. tonsa* egg or nauplii. The copepod *A. royi* contains also great nutritional values, however, the use of this species for larvae feeding could be limited by their fast swimming behavior and high ability of predator detection, and its use as live prey should be carefully evaluated/ decided by considering the swimming ability of fish larvae of specific species. The feeding suitability of copepods for fish larvae are species-specific, other trials would be expected to carry out in the coming studies with the thorough understanding of the fish larvae feeding behavior and nutritional requirements.

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Appendix 1- Curriculum Vitae

Yen-Ju Pan
Co-tutorial Ph.D. Student
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Personal Information

Name : Yen-Ju Pan
Nationality : Taiwanese
Birthday : 11.07.1988
Permanent address : 4F., No.7, Aly. 9, Ln. 200, Guangming St., Xindian Dist., New Taipei City 231, Taiwan (R.O.C.)
Language skills : Mandarin Chinese, English, Basic French

Educational background

2006~2010 : Bachelor of Science, Department of Biology
National Changhua University of Education, Taiwan R.O.C.
Major: Biology and Education Program for Secondary School Teachers

2010~2012 : Master of Science, Institute of Marine Biology
National Taiwan Ocean University, Taiwan R.O.C.
Major: Marine Biology

2012~now : Ph.D. program of Institute of Marine Biology
National Taiwan Ocean University, Taiwan R.O.C.
Expected graduation year: 2016

2013~now : Enrolled in the co-tuturial Ph.D. program of Université Lille 1
Expected graduation year: 2016

Research interest

I am broadly interested in biological responses of animals under different environmental conditions. During my master and doctoral studies, I focused on how the diets and environmental conditions affect the reproduction, morphology, fatty acid profile and egg dormancy of calanoid and cyclopoid copepods. My work aims to release the ecological and biological understandings on copepods, and further apply

this knowledge for aquaculture applications.

Professional skills

- Cultivation techniques of copepods, phytoplankton and fish larvae
- Scanning electron microscope (SEM)
- Transmission electron microscopy (TEM)
- GC-MS analysis (fatty acid profiling)
- Basic skills for molecular study (DNA, RNA extractions, PCR and electrophoresis)
- Certified NAUI open water diver and SSI Advanced diver

Certificate of Language

- TOEIC English Examine: 890
- IELTS English Examine: Overall band 6.5
- French diploma (DELF): Level A1

Awards and Scholarships

- 2013-2015 Joseph Fourier scholarship from Bureau François de Taipei (24 months)
- 2013 Co-tutorial thesis scholarship from International division of Université Lille 1 (4 months)
- 2013 Co-funding professional training scholarship from Doctoral school ED SMRE of Université Lille 1 (1 week extensive aquaculture training in University of Las Palmas de Gran Canaria)
- 2014 Overseas Project for Post Graduate Research from National Science Council of Taiwan (8 months)
- 2015-2016 Eiffel Excellence Scholarship from Ministry of Foreign Affairs of France (10 months)

Publications

Communicates of International Conference and workshop

Yen-Ju Pan*, Sami Souissi, Anissa Souissi, Cheng-Han Wu, Shin-Hong Cheng, Jiang-Shiou Hwang, 2012. The dietary effects on the egg production, egg hatching rate and female life span of the tropical calanoid copepod *Acartia bilobata*. The Second Cross-Straits Workshop on Marine Biodiversity, Keelung, Taiwan.

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Yen-Ju Pan*, Anissa Souissi, Jiang-Shiou Hwang, Sami Souissi, 2013. The effects of algal diet on the egg production, female life span; and the hatching success rate of the cold-storage egg of *Acartia bilobata*. Aquaculture conference: To the Next 40 Years of Sustainable Global Aquaculture, Las Palmas de Gran Canaria, Spain.

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Yen-Ju Pan*, Emilien Déposé, Edouard Husson, Stéphane Hénard, Dominique Mallevoye, Frédéric Cousin, Anissa Souissi, Jiang-Shiou Hwang, Sami Souissi, 2014. The application of copepod in aquaculture: examples from the free spawning and the egg bearing copepods. PHC- STAR France-Korea workshop in Evaluation of ecotoxicological biomarkers and assays, Sungkyunkwan University, Korea.

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Pan, Y. J.*, Souissi, S., Souissi, A., Wu, C. H., Cheng, S. H., & Hwang, J. S. (2014). Dietary effects on egg production, egg-hatching rate and female life span of the tropical calanoid copepod *Acartia bilobata*. *Aquaculture Research*, **45(10)**, 1659-1671.

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Pan, Y. J. *, Souissi, A., Hwang, J. S., & Souissi, S. (in press). Artificially cold-

induced quiescent egg viability of the tropical copepod *Acartia bilobata* (Copepoda, Calanoida). *Aquaculture Research*. DOI: 10.1111/are.12968

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Appendix 2- Manuscript 1

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Appendix 3- Manuscript 2

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